SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

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División de Nuevas Creaciones

SOLICITUD PCT

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ENTRADA EN FASE NACIONAL

21. **EXPEDIENTE No.** 07-136194

54. **TITULO** EVENTO MON89788 DE SOYA Y METODOS PARA LA DETECCION DEL MISMO

51. **CLASIFICACION INTERNACIONAL**

71. **SOLICITANTE** MONSANTO TECHNOLOGY LLC

DOMICILIO *ST. LOUIS, MO, ESTADOS UNIDOS DE AMÉRICA*

74. **APODERADO** JUAN PABLO CADENA SARMIENTO

22. **SANTAFE DE BOGOTA, D.C.**

PETITORIO

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Industria y Comercio
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27 dic 2007

1 SOLICITUD DE:

☒ Patente de Invención. ☐ Patente de Modelo de Utilidad.

☒ Capítulo I. ☐ Capítulo II.

2 SOLICITANTE (71)	Nombre: **MONSANTO TECHNOLOGY LLC** Dirección: 800 North Lindbergh Boulevard, St. Louis, Missouri 63167, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América Lugar de Constitución: Estados Unidos de América Teléfono: Fax: E-mail	**IDENTIFICACIÓN** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número _____
3 REPRESENTANTE O APODERADO	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Cra. 7 No. 71-21 Torre B – Piso 4, Bogotá, Colombia. Teléfono: 744-2200 Fax: 742-2200 E-mail:	**IDENTIFICACIÓN** C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número 80.410.337 TP 57492
4 INVENTOR (ES) (72)	**Nombre:** Marianne MALVEN, Jennifer RINEHART, Nancy TAYLOR, Ellen DICKINSON. **Dirección:** 800 North Lindbergh Blvd., St. Louis, MO 63167, US; 800 North Lindberg Blvd., St. Louis, MO 63167, US; 800 North Lindbergh Blvd., St. Louis, MO 63167, US; 800 North Lindbergh Blvd., St. Louis, MO 63167, US (Respectivamente). **Nacionalidad o Domicilio:** Estados Unidos de América (Respectivamente).	

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Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

10 **ANEXOS**

- [x] Comprobante de pago de la tasa de presentación de la solicitud. $482.000
- [x] Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- [x] Poderes, si fuere el caso. **Fotocopia autenticada**
- [ ] Documento de cesión del inventor al solicitante o a su causante.
- [ ] Declaraciones.
- [x] Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- [x] Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
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- [x] Otros: Tarjeta de Propietarios
  Listado de Secuencias
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11 FIGURA CARACTERÍSTICA:

12 Solicito la concesión de la patente,

NOMBRE: JUAN PABLO CADENA SARMIENTO

FIRMA: _____

C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

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***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	482,000.00
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PATENTE [X] PCT/US2006/020323 **MODELO DE UTILIDAD** [ ] **DISEÑO INDUSTRIAL** [ ]

SOLICITANTE/S. RAZON SOCIAL O APELLIDOS Y NOMBRES MONSANTO TECHNOLOGY LLC

DOMICILIO ST. LOUIS, MO, ESTADOS UNIDOS DE AMERICA

APODERADO JUAN PABLO CADENA SARMIENTO

TITULO EVENTO MON89788 DE SOYA Y METODOS PARA LA DETECCION DEL MISMO

CLASIFICACION

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PRORROGA CAMBIO DE NOMBRE

TRASPASO A

INVENTOR (ES) MARIANNE MALVEN, JENNIFER RINEHART, NANCY TAYLOR, ELLEN DICKINSON.

OTROS

OFICINA DE COMUNICACIONES

INTERNATIONAL BUREAU OF WIPO

In re Application of:
Monsanto Technology LLC

Serial No.: PCT/US2006/020323

Atty. Dkt. No.: MONS:113WO

Filed: May 26, 2006

For: Soybean Event MON89788 and Methods for Detection Thereof

REQUEST TO CORRECT FACE SHEET OF PUBLICATION

Via Facsimile No. 011 41 22 338 82 70

International Bureau
WIPO
34, chemin des Colombettes
1211 Geneva 20
Switzerland

Dear Madam or Sir:

Please correct the international filing date in item (22) on the face sheet of the international publication to read May 26, 2006. Attached is a copy of the filing receipt evidencing the filing date of May 26, 2006.

Should this Authority have any questions regarding this matter, please contact the undersigned.

Respectfully submitted,

Robert E. Hanson, Agent

Sonnenschein Nath & Rosenthal LLP
1717 Main Street, Suite 3400
Dallas, TX 75201
Telephone No.: 214.259.0931
Facsimile No.: 214.259.0910

Date: November 12, 2007

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PATENT COOPERATION TREATY

From the RECEIVING OFFICE

To:
ROBERT E. HANSON
FULBRIGHT & JAWORSKI L.L.P.
600 CONGRESS AVENUE, SUITE 2400
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PCT

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

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Date of mailing *(day/month/year)*	08 Aug 2006
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Applicant: MONSANTO TECHNOLOGY LLC

Title of the invention: SOYBEAN EVENT MON89788 AND METHODS FOR DETECTION THEREOF

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

☐ was transmitted to the International Bureau on 08 Aug 2006

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(71) Applicant *(for all designated States except US)*: **MONSANTO TECHNOLOGY LLC** [—/US]; 800 North Lindbergh Blvd., St. Louis, MO 63167 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MALVEN, Marianne** [—/US]; 800 North Lindbergh Blvd., St. Louis, MO 63167 (US). **RINEHART, Jennifer** [—/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US). **TAYLOR, Nancy** [—/US]; 800 North Lindbergh Blvd., St. Louis, MO 63167 (US). **DICKINSON, Ellen** [—/US]; 800 North Lindbergh Blvd., St. Louis, MO 63167 (US).

(74) Agent: **HANSON, Robert, E.**; FULBRIGHT & JAWORSKI L.L.P., 600 Congress Avenue, Suite 2400, Austin, TX 78701 (US).

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(54) Title: SOYBEAN EVENT MON89788 AND METHODS FOR DETECTION THEREOF

(57) Abstract: The present invention provides for soybean plant and seed comprising transformation event MON89788 and DNA molecules unique to these events. The invention also provides methods for detecting the presence of these DNA molecules in a sample.

DESCRIPTION

SOYBEAN EVENT MON89788 AND METHODS FOR DETECTION THEREOF

BACKGROUND OF THE INVENTION

This application claims priority from U.S. Provisional Application No. 60/685,584, filed May 27, 2005, the entire disclosure of which is herein incorporated by reference.

1. Field of the Invention

The present invention relates to a new and distinctive transgenic soybean transformation event, designated MON89788, a soybean cultivar derived therefrom, and plant parts, seed, and products thereof. The invention also relates to assays for detecting the presence of a DNA molecule specific to MON89788 in a plant part extract or seed extract.

2. Description of Related Art

Soybean (*Glycine max*) is an important crop in many areas of the world. The methods of biotechnology have been applied to soybean for improvement of the agronomic traits and the quality of the product. One such agronomic trait important in soybean production is herbicide tolerance, in particular, tolerance to glyphosate herbicide. A herbicide tolerant soybean event would be a useful trait for managing weeds.

N-phosphonomethylglycine, also known as glyphosate, is a well-known herbicide that has activity on a broad spectrum of plant species. Glyphosate is the active ingredient of Roundup® (Monsanto Co., St. Louis, MO), a safe herbicide having a desirably short half-life in the environment. When applied to a plant surface, glyphosate moves systemically through the plant. Glyphosate is phytotoxic due to its inhibition of the shikimic acid pathway, which provides a precursor for the synthesis of aromatic amino acids. Glyphosate inhibits the enzyme 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS) found in plants.

Glyphosate tolerance can be achieved by the expression of EPSPS variants that have lower affinity for glyphosate and therefore retain their catalytic activity in the presence of glyphosate (U.S. Patent Nos. 5,633,435; 5,094,945; 4,535,060, and 6,040,497). Enzymes that degrade glyphosate in plant tissues (U.S. Patent No. 5,463,175) are also capable of conferring cellular tolerance to glyphosate. Such genes are used for the production of transgenic crops that are tolerant to glyphosate, thereby allowing glyphosate to be used for effective weed control with minimal concern of crop damage. For example, glyphosate tolerance has been genetically engineered into corn (U.S. Patent No. 5,554,798), wheat (U.S. Patent 6,689,880), cotton (U.S. Patent 6,740,488), soybean (WO 9200377) and canola (US Patent Appl. 20040018518). The transgenes for glyphosate tolerance and the transgenes for tolerance to other herbicides, *e.g.* the *bar* gene, (Toki *et al.*, 1992; Thompson *et al.*, 1987; phosphinothricin acetyltransferase (DeBlock *et al.*, 1987), for tolerance to glufosinate herbicide) are also useful as selectable markers or scorable markers and can provide a useful phenotype for selection of plants linked with other agronomically useful traits.

The expression of foreign genes in plants is known to be influenced by their chromosomal position, perhaps due to chromatin structure *(e.g.*, heterochromatin) or the proximity of transcriptional regulation elements (*e.g.*, enhancers) close to the integration site (Weising *et al.*, 1988). For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure

reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example. It is possible to detect the presence of a transgene by any well known polynucleic acid detection method such as the polymerase chain reaction (PCR) or DNA hybridization using polynucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct unless the sequence of chromosomal DNA ("flanking DNA") adjacent to the inserted transgene DNA is known. An event-specific PCR assay is discussed, for example, by Windels *et al.* (1999), who identified glyphosate tolerant soybean event 40-3-2 by PCR using a primer set spanning the junction between the insert transgene and flanking DNA, specifically one primer that included sequence from the insert and a second primer that included sequence from flanking DNA. Transgenic plant event specific DNA detection methods have also been described in US Patent Nos. 6,893,826; 6,825,400; 6,740,488; 6,733,974 and 6,689,880; 6,900,014 and 6,818,807, herein incorporated by reference in their entirety.

This invention relates to the glyphosate tolerant soybean event MON89788 (also referred to as MON19788 or GM_A19788) and to the DNA molecules contained in these soybean plants that are useful in detection methods for the plant and progeny thereof and plant tissues derived from MON89788.

SUMMARY OF THE INVENTION

The present invention provides a soybean transgenic event designated MON89788 (also referred to as MON19788) and progeny thereof having representative seed deposited with American Type Culture Collection (ATCC) with accession No. PTA-6708. Another aspect of the invention is the plant cells or regenerable parts of the plant and seeds of the soybean event MON89788. The invention also includes plant parts of soybean event

MON89788 that include, but are not limited to a cell, pollen, ovule, flowers, shoots, roots, leaves, and products derived from MON89788, for example soybean meal, flour and oil.

One aspect of the invention provides compositions and methods for detecting the presence of a DNA transgene/genomic junction region from a soybean event MON89788 plant or seed or products derived from plant parts or seed. DNA molecules are provided that comprise at least one transgene/genomic junction DNA molecule selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, and complements thereof, wherein the junction molecule spans the insertion site that comprises a heterologous DNA inserted into the genome of the soybean cell and the genomic DNA from the soybean cell flanking the insertion site soybean event MON89788. Such junction sequences may, in one aspect of the invention, be defined as comprising nucleotides 1093-1113 or 5396-5416 of SEQ ID NO:9, respectively. In other aspects of the invention, the junctions may be defined as including additional portions of the flanking genome and transgene, for example, and may be defined as comprising one or more sequence as given by nucleotides 1073-1113, 1043-1113, 1093-1133, 1093-1163, 1043-1163, 5376-5416, 5346-5416, 5396-5436, 5396-5416, 5396-5466, or 5346-5466 of SEQ ID NO:9. Such sequences and plants and seeds comprising these sequences therefore form one aspect of the invention.

A novel DNA molecule is provided that is a DNA transgene/genomic region SEQ ID NO:3 or the complement thereof, from soybean event MON89788. A soybean plant and seed comprising SEQ ID NO:3 in its genome is an aspect of this invention. SEQ ID NO:3 further comprises SEQ ID NO:1 in its entirety.

According to another aspect of the invention, a DNA molecule is provided that is a DNA transgene/genomic region SEQ ID NO:4, or the complement thereof, wherein this DNA molecule is novel in soybean event MON89788. A soybean plant and seed comprising SEQ ID NO:4 in its genome is an aspect of this invention. SEQ ID NO:4 further comprises SEQ ID NO:2 in its entirety.

According to another aspect of the invention, two nucleic acid molecules are provided for use in a DNA detection method, wherein the first nucleic acid molecule comprises at least 11 or more contiguous polynucleotides of any portion of the transgene region of the DNA molecule of SEQ ID NO:3 and the second nucleic acid is a molecule of similar length of any portion of a 5' flanking soybean genomic DNA region of SEQ ID NO:3, wherein these nucleic acid molecules when used together are useful as primers in a

DNA amplification method that produces an amplicon. The amplicon produced using these primers in the DNA amplification method is diagnostic for soybean event MON89788 DNA. The amplicon produced by the described primers that is homologous or complementary to a portion of SEQ ID NO:3 comprising SEQ ID NO:1 is an aspect of the invention.

According to another aspect of the invention, two nucleic acid molecules are provided for use in a DNA detection method, wherein the first nucleic acid molecule comprises at least 11 or more contiguous polynucleotides of any portion of the transgene region of the DNA molecule of SEQ ID NO:4 and a second nucleic acid molecule of similar length of any portion of a 3' flanking soybean genomic DNA of SEQ ID NO:4, wherein these nucleic acid molecules when used together are useful as primers in a DNA amplification method that produces an amplicon. The amplicon produced using these primers in the DNA amplification method is diagnostic for soybean event MON89788 DNA. The amplicon produced by the described primers that is homologous or complementary to a portion of SEQ ID NO:4 comprising SEQ ID NO:2 is an aspect of the invention.

Any nucleic acid primer pair derived from SEQ ID NO:3 or SEQ ID NO:4, or SEQ ID NO:9 or the complements thereof, that when used in a DNA amplification reaction produces an amplicon diagnostic for soybean event MON89788-derived tissue, such as an amplicon that comprises SEQ ID NO:1 or SEQ ID NO:2 or any portion of SEQ ID NO:9 respectively, is another embodiment of the invention. In a particular embodiment, the primer pair may consist of primer A (SEQ ID NO:5) and primer D (SEQ ID NO:8).

Another aspect of the invention is a soybean plant, or seed, or product derived from a plant or seed comprising event MON89788, in which the genomic DNA when isolated from the soybean plant, or seed, or product produces an amplicon in a DNA amplification method that comprises SEQ ID NO:1 or SEQ ID NO:2.

Still another aspect of the invention is a soybean plant, or seed, or product derived from a plant or seed comprising MON89788, in which the genomic DNA when isolated from the soybean plant, or seed, or product produces an amplicon in a DNA amplification method, wherein DNA primer molecules SEQ ID NO:5 and SEQ ID NO:6 are used in the DNA amplification method.

Yet another aspect of the invention is a soybean plant, seed, product, or commodity derived from the plant or seed, comprising MON89788, in which the genomic DNA when isolated from the soybean plant, or seed, or product produces an amplicon in a DNA amplification method, wherein DNA primer molecules SEQ ID NO:7 and SEQ ID NO:8 are used in the DNA amplification method. The product or commodity may comprise, without limitation, a food or feed product comprising or derived from one or more of the following products of a soybean plant comprising event MON89788: lecithin, fatty acids, glycerol, sterol, edible oil, defatted soy flakes, soy meals including defatted and toasted soy meals, soy milk curd, tofu, soy flour, soy protein concentrate, isolated soy protein, hydrolyzed vegetable protein, textured soy protein, and soy protein fiber.

According to another aspect of the invention, a method of detecting the presence of DNA corresponding specifically to the soybean event MON89788 DNA in a sample is provided. Such method comprising: (a) contacting a sample comprising DNA with a DNA primer pair; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon, wherein said amplicon comprises SEQ ID NO:1 or SEQ ID NO:2. A kit comprising DNA primer molecules that when used in a DNA amplification method produces an amplicon comprising SEQ ID NO:1 or SEQ ID NO:2 is a further aspect of the invention.

According to another aspect of the invention, a method of detecting the presence of DNA corresponding specifically to the soybean event MON89788 DNA in a sample is provided. Such method comprising: (a) contacting a sample comprising DNA with a probe that hybridizes under stringent hybridization conditions with genomic DNA from soybean event MON89788 and does not hybridize under the stringent hybridization conditions with a control soybean plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the soybean event MON89788 DNA, wherein said probe comprises SEQ ID NO:1 or SEQ ID NO:2. The sample may comprise a progeny seed, plant, or plant part comprising soybean event MON89788, or any of the following products derived from a plant comprising MON89788: lecithin, fatty acids, glycerol, sterol, edible oil, defatted soy flakes, soy meals including defatted and toasted soy meals, soy milk curd, tofu, soy flour, soy protein concentrate, isolated soy protein, hydrolyzed vegetable protein, textured soy protein, and soy protein fiber. A kit comprising a DNA probe comprising a DNA molecule that is

homologous or complementary to SEQ ID NO:1 or SEQ ID NO:2 is an aspect of the invention. A kit comprising a DNA molecule comprising SEQ ID NO:18, SEQ ID NO:19, or SEQ ID NO:20, or their complements, is also an aspect of the invention.

According to another aspect of the invention, a method of producing a soybean plant that tolerates an application of glyphosate is provided that comprise the steps of: (a) sexually crossing a first parental glyphosate tolerant soybean plant comprising event MON89788, and a second parental soybean plant that lacks the glyphosate tolerance, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that tolerates application of glyphosate. Breeding methods may additionally comprise the steps of crossing the parental plant comprising soybean event MON89788 to a second parental soybean plant that is also tolerant to glyphosate and selecting for glyphosate tolerant progeny by molecular marker DNA genetically linked to the glyphosate tolerant phenotype found in each parent.

Another aspect of the invention is a method to control weeds in a field of soybean plants comprising MON89788, wherein said method comprises planting a field with soybean seed comprising event MON89788 said representative seed deposited as ATCC accession No. PTA-6708, allowing said seed to germinate and treating said plants with an effective dose of glyphosate to control weed growth in said field.

The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1. Organization of the transgene insertion in the genome of a soybean plant comprising event MON89788.

FIG. 2A- 2B. Processing of commodity products from soybean.

DETAILED DESCRIPTION

The present invention relates to a novel soybean transformation event designated MON89788 that provides glyphosate tolerance, and the plant parts and seed and products produced from plants, plant parts, seed, and products comprising the event. The invention provides DNA molecules that are novel in the genome of soybean cells comprising MON89788 and DNA molecules that can be used in various DNA detection methods to

identify MON89788 DNA in a sample. The invention provides a method to control weeds in a field of plants containing MON89788 by treating the weeds in the field comprising plants comprising event MON89788 with a glyphosate herbicide.

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger *et al.* (1991) and Lewin (1994). The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used.

As used herein, the term "soybean" means *Glycine max* and includes all plant varieties that can be bred with soybean.

As used herein, the term "comprising" means "including but not limited to".

"Glyphosate" refers to N-phosphonomethylglycine and its salts, Glyphosate is the active ingredient of Roundup® herbicide (Monsanto Co.). Treatments with "glyphosate herbicide" refer to treatments with the Roundup®, Roundup Ultra®, Roundup Pro® herbicide or any other herbicide formulation containing glyphosate. Examples of commercial formulations of glyphosate include, without restriction, those sold by Monsanto Company as ROUNDUP®, ROUNDUP® ULTRA, ROUNDUP® ULTRAMAX, ROUNDUP® CT, ROUNDUP® EXTRA, ROUNDUP® BIACTIVE, ROUNDUP® BIOFORCE, RODEO®, POLARIS®, SPARK® and ACCORD® herbicides, all of which contain glyphosate as its isopropylammonium salt; ROUNDUP® WEATHERMAX (glyphosate potassium salt), those sold by Monsanto Company as ROUNDUP® DRY and RIVAL® herbicides, which contain glyphosate as its ammonium salt; that sold by Monsanto Company as ROUNDUP® GEOFORCE, which contains glyphosate as its sodium salt; and that sold by Syngenta Crop Protection as TOUCHDOWN® herbicide, which contains glyphosate as its trimethylsulfonium salt. Treatment of a field comprising glyphosate tolerant soybean plants comprising event MON89788 with any of these glyphosate herbicide formulations will control weed growth in the field and not affect the growth or yield of the soybean plants comprising MON89788.

A transgenic "event" is produced by transformation of plant cells with heterologous DNA, for example, a nucleic acid construct that includes a transgene of

interest, regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous transgene DNA and the flanking genomic DNA. The term "event" also refers to DNA from the original transformant comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (for example, the original transformant and progeny resulting from the selfing) and a parental line that does not contain the inserted DNA.

A glyphosate tolerant soybean plant can be bred by first sexually crossing a first parental soybean plant consisting of a soybean plant grown from a transgenic glyphosate tolerant soybean plant comprising MON89788 or an soybean plant that is a progeny of the cross of such a plant that expresses the glyphosate tolerant phenotype, and a second parental soybean plant that lacks the tolerance to glyphosate, thereby producing a plurality of first progeny plants; and then selecting a progeny plant that is tolerant to application of glyphosate herbicide. These steps can further include the back-crossing of the glyphosate tolerant progeny plant to the second parental soybean plant or a third parental soybean plant, then selecting progeny by application with glyphosate or by identification with molecular markers associated with the trait thereby producing an soybean plant that tolerates the application of glyphosate herbicide. Molecular markers may be used that comprise the junction DNA molecules identified at the 5' and 3' sites of insertion of the transgene in event MON89788.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating, exogenous transgenes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant as previously described is also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, *e.g.*, Fehr, (1987).

A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, for example, a radioactive isotope, a ligand, a chemiluminescent agent, or an enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of genomic DNA from a soybean plant comprising event MON89788 whether from a soybean plant or seed or from a sample or extract of the plant or seed that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids, but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

"Primers" are isolated polynucleic acids that are annealed to a complementary target polynucleic acid strand by nucleic acid hybridization to form a hybrid between the primer and the target polynucleic acid strand, then extended along the target polynucleic acid strand by a polymerase, for example, a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target polynucleic acid molecule, for example, by the polymerase chain reaction (PCR) or other conventional nucleic acid amplification methods.

Probes and primers are generally 11 polynucleotides or more in length, preferably 18 polynucleotides or more, more preferably 24 polynucleotides or 30 polynucleotides or more. Such probes and primers hybridize specifically to a target molecule under high stringency hybridization conditions. Preferably, probes and primers according to the present invention have complete sequence identity with the target molecule, although probes differing from the target sequence and that retain the ability to hybridize to target sequences under high stringency conditions may be designed by conventional methods.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.* (1989); Ausubel *et al.* (1992); and Innis *et al.* (1990). PCR-primer pairs (a primer set) can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Primers and probes based on the flanking genomic DNA and insert sequences disclosed herein (SEQ ID NOs:1-4 and 9) can be used to confirm and, if necessary, to correct the disclosed sequences by conventional methods, for example, by isolating the corresponding DNA molecule from a deposit of seed comprising MON89788, and

determining the nucleic acid sequence such molecules. Additional associated DNA molecules may be isolated from the genome of a cell comprising MON89788 that comprise the transgene insert and genomic flanking regions, and fragments of these molecules may be used as primers or probes.

The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from the MON89788 event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure and are of sufficient length to maintain this structure under high stringency conditions. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook *et al.*, 1989, and by Haymes *et al.* (1985). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

As used herein, a substantially homologous sequence is a nucleic acid sequence that will specifically hybridize to the complement of the nucleic acid sequence to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate

up to 42 kb of bacteriophage DNA (Cheng *et al.*, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. The sequence of the heterologous DNA insert or flanking sequence from soybean event MON89788 and can be verified, and corrected if necessary by amplifying such molecules from the event genome using primers derived from the sequences provided herein followed by standard DNA sequencing methods applied to the PCR amplicon or to isolated cloned transgene/genomic DNA.

The amplicon produced by these methods may be detected by a plurality of techniques. One such method is Genetic Bit Analysis (Nikiforov, *et al.*, 1994) where an DNA oligonucleotide is designed which overlaps both the adjacent flanking genomic DNA sequence and the inserted DNA transgene sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking genomic sequence), a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labelled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking genomic sequence due to successful amplification, hybridization, and single base extension.

Another method is the Pyrosequencing technique as described by Winge (2000). In this method an oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. DNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization as described by Chen *et al.* (1999) is a method that can be used to detect the amplicon of the present invention. Using this method an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic DNA

sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking genomic sequence due to successful amplification, hybridization, and single base extension.

Taqman® (PE Applied Biosystems, Foster City, CA) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the genomic flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking genomic /transgene insert sequence due to successful amplification and hybridization.

Molecular Beacons have been described for use in sequence detection as described in Tyangi *et al.* (1996) Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking genomic/transgene insert sequence due to successful amplification and hybridization.

Other described methods, such as, microfluidics (US Patent Pub. 2006068398, US Patent No. 6,544,734) provide methods and devices to separate and amplify DNA samples. Optical dyes used to detect and quantitate specific DNA molecules (WO/05017181). Nanotube devices (WO/06024023) that comprise an electronic sensor for the detection of DNA molecules or nanobeads that bind specific DNA molecules and can then be detected are useful for detecting DNA molecules of the present invention.

DNA detection kits can be developed using the compositions disclosed herein and the methods described or known in the art of DNA detection. The kits are useful for the identification of soybean event DNA in a sample and can be applied to methods for breeding soybean plants containing DNA. The kits may contain DNA primers or probes that are homologous or complementary to SEQ ID NOs:1-4 and 9 or DNA primers or probes homologous or complementary to DNA contained in the transgene genetic elements of DNA, these DNA sequences can be used in DNA amplification reactions or as probes in a DNA hybridization method. The structure of the DNA of the transgene genetic elements contained in the soybean genome and illustrated in Figure 1 comprises a 5' genomic region of the soybean A3244 genome flanking the transgene insert, the insert comprising a portion of the right border region (RB) from *Agrobacterium tumefaciens*, the chimeric promoter FMV/Tsf1 and related linked elements (US Patent 6,660,911; also referred to as FMV/E1F1α) is operably connected to an *Arabidopsis* EPSPS chloroplast transit peptide coding sequence (herein referred to as CTP2 or TS-AtEPSPS CTP2, US Patent 5,633,435, operably connected to a glyphosate resistant EPSPS (herein referred to as CP4 EPSPS or aroA:CP4, isolated from *Agrobacterium tumefaciens* strain CP4 and coding sequence modified for enhanced expression in plant cells, U.S. Patent 5,633,435), operably connected to the 3' termination region from pea ribulose 1,5-bisphosphate carboxylase (herein referred to as E9 3' or T-Ps.RbcS:E9, Coruzzi *et al.*, (1984), a portion of the left border (LB) region from *Agrobacterium tumefaciens*, and the 3' genomic region of the soybean A3244 genome flanking the transgene insert. DNA molecules useful as primers in DNA amplification methods can be derived from the sequences of the genetic elements of the transgene insert contained in soybean event MON89788. These primer molecules can be used as part of a primer set that also includes a DNA primer molecule derived from the genome of soybean flanking the transgene insert. Soybean event MON89788 was produced by transformation of soybean line A3244 (US Patent 5,659,114) by an *Agrobacterium* mediated method, for example, methods described in US Patents 6,384,301 and 7,002,058 (herein incorporated by reference in their entirety).

The inventors of the present invention have discovered that a soybean line comprising the MON89788 T-type genomic region (T-type is combination of a transgene and the associated haplotype region of a plant genome) in its genome has an improved yield relative to a line comprising the previous 40-3-2 T-type genomic region. This was

demonstrated in replicated field trials including yield data collected from multiple locations in the United States (US patent application 60/685584).

The following examples are included to demonstrate examples of certain preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1

Production of Amplicon Diagnostic for MON89788 Genomic DNA

DNA from transgenic soybean event MON89788 is extracted from tissue comprising soybean seeds, vegetative tissue, or meal. The DNA is isolated from the tissue using Qiagen's DNeasy Plant Miniprep Kit according to the manufacturer's instructions (Qiagen Corp. Valencia, CA).

A PCR product is produced that comprises a portion of the genomic DNA flanking the 5' end of the T-DNA (transfer DNA comprising the transgene) insertion in the genome of a plant comprising MON89788. This DNA product comprises SEQ ID NO:3. The PCR may be performed using one primer designed to hybridize to the genomic DNA sequences flanking the 5' end of the transgene insert (DNA primer A, SEQ ID NO:5; see FIG. 1) paired with a second primer (DNA primer B, SEQ ID NO:6) located in the transgene promoter region (US Patent 6,660,911, SEQ ID NO:28, herein incorporated by reference and found within SEQ ID NO:9).

A PCR product is produced from the 3' end of the transgene insert that comprises a portion of the genomic DNA flanking the 3' end of the T-DNA insertion in the genome of a plant comprising MON89788 This DNA product comprises SEQ ID NO:4. PCR may be performed using one primer designed to hybridize to the genomic DNA sequences flanking the 3' end of the insert of each event (DNA primer D, SEQ ID NO:8) and paired

with a second primer (DNA primer C, SEQ ID NO:7) located in the T-Ps.RbcS:E9 3' transcription termination sequence at the 3' end of the insert.

The PCR template includes ~50 ng of genomic DNA. As a negative control ~50 ng of genomic DNA from the non-transgenic soybean cultivar is utilized. Each PCR reaction contains 5 µl 10 X Buffer for REDAccuTaq™ LA DNA Polymerase Mix (Sigma-Aldrich, St Louis, MO), 200 µM each dNTP (Sigma-Aldrich), 0.4 µM each primer, and 2.5 Units JumpStart™ REDTaq™ DNA Polymerase (Sigma-Aldrich) in a 50 µl total volume reaction. The PCR reactions are performed under the following cycling conditions: 1 cycle at 94°C for 3 minutes (min); 32 or 35 cycles at 94°C for 30 seconds (s), 58°C for 30 s, 72°C for 30 s or 1 min; 1 cycle at 72°C for 10 min.

DNA event primer pairs are used to produce an amplicon diagnostic for MON89788 genomic DNA. These event primer pairs include, but are not limited to primers A and B (SEQ ID NO:5 and 6) and event primer pairs C and D (SEQ ID NO: 7 and 8), that are used in the described DNA amplification method. In addition to these primer pairs, any primer pair derived from SEQ ID NO:3 or SEQ ID NO:4, or the complements thereof, that when used in a DNA amplification reaction produces an amplicon that comprises SEQ ID NO:1 or SEQ ID NO:2 diagnostic for soybean MON89788 event-derived tissue, respectively, may be utilized. DNA amplification conditions illustrated in Table 1 and Table 2 can be used to produce a diagnostic amplicon for MON89788 using the appropriate event primer pairs. Any modification of these methods used to produce an amplicon diagnostic for MON89788 is within the ordinary skill of the art. An extract putatively containing DNA of a soybean plant or seed comprising MON89788, or a product derived from a plant comprising MON89788 that when tested in a DNA amplification method produces an amplicon diagnostic for soybean event MON89788 may be utilized as a template for amplification to determine whether MON89788 is present.

The amplicon is produced by the use of at least one primer sequence derived from SEQ ID NO:3 or SEQ ID NO:4 that when used in a PCR method produces a diagnostic amplicon for event MON89788. For example, the production of the MON89788 amplicons can be performed using a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler as shown in Table 2, or by methods and apparatus known to those skilled in the art.

Table 1. PCR procedure and reaction mixture conditions for the identification of soybean MON89788 5' transgene insert/genomic junction region.

Step	Reagent	Amount	Comments
1	Nuclease-free water	add to final volume of 20 µl	-
2	10X reaction buffer (with $MgCl_2$)	2.0 µl	1X final concentration of buffer, 1.5 mM final concentration of $MgCl_2$
3	10 mM solution of dATP, dCTP, dGTP, and dTTP	0.4 µl	200 µM final concentration of each dNTP
4	Event primer A (SEQ ID NO:5 resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.2 µl	0.1 µM final concentration
5	Event primer B (SEQ ID NO:6 resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.2 µl	0.1 µM final concentration
6	RNase, DNase free (500 µg/ml)	0.1 µl	50 ng/reaction
7	*REDTaq* DNA polymerase (1 unit/µl)	1.0 µl (recommended to switch pipets prior to next step)	1 unit/reaction
8	Extracted DNA (template): • Samples to be analyzed: * individual leaves * pooled leaves (maximum of 10 leaves/pool) • Negative control • Negative control • Positive control	 • 10-200 ng of genomic DNA • 200 ng of genomic DNA • 50 ng of non-transgenic soybean genomic DNA • no template DNA (solution in which DNA was resuspended) • 50 ng of soybean genomic DNA comprising MON89788	-

Gently mix and, if needed (no hot top on thermocycler), add 1-2 drops of mineral oil on top of each reaction. Proceed with the PCR in a Stratagene Robocycler (Stratagene, La Jolla, CA), MJ Engine (MJR-Biorad, Hercules, CA), Perkin-Elmer 9700 (Perkin Elmer, Boston, MA), or Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) thermocycler using the following cycling parameters (Table 2). The MJ Engine

or Eppendorf Mastercycler Gradient thermocycler should be run in the calculated mode. Run the Perkin-Elmer 9700 thermocycler with the ramp speed set at maximum.

Table 2. Thermocycler conditions

Cycle No.	Settings: Stratagene Robocycler	
1	94°C	3 minutes
34	94°C	1 minute
	64°C	1 minute
	72°C	1 minute and 30 seconds
1	72°C	10 minutes

Cycle No.	Settings: MJ Engine or Perkin-Elmer 9700	
1	94°C	3 minutes
34	94°C	30 seconds
	64°C	30 seconds
	72°C	1 minute
1	72°C	10 minutes

Cycle No.	Settings: Eppendorf Mastercycler Gradient	
1	94°C	3 minutes
34	94°C	15 seconds
	64°C	15 seconds
	72°C	1 minute
1	72°C	10 minutes

EXAMPLE 2

Sequence Determination of Transgene/Genomic Region and Southern Analysis

DNA sequencing of the PCR products provides for DNA that can be used to design additional DNA molecules as primers and probes for the identification of soybean plants or seed comprising MON89788. PCR products of the expected sizes representing the 5' and 3' transgene/genomic sequences were isolated by separation of the PCR products on a 2.0% agarose gel by electrophoresis. PCR products are isolated that include the 5' and 3' DNA regions that span the insert junction between the transgene insertion into the soybean genome. The 5' and 3' PCR products for MON89788 are purified by agarose gel electrophoresis followed by isolation from the agarose matrix using the QIAquick Gel Extraction Kit (catalog # 28704, Qiagen Inc., Valencia, CA). The purified PCR products are then sequenced (*e.g.* ABI Prism™ 377, PE Biosystems, Foster City, CA) and analyzed (*e.g.* DNASTAR sequence analysis software, DNASTAR Inc., Madison, WI).

A DNA sequence was determined for the nucleotide base pair segment representing the transgene/genomic region of event MON89788 as illustrated in FIG 1 and identified as SEQ ID NO:9.. The genomic and transgene elements that are contained in SEQ ID NO:9 are described in Table 3. The 5' and 3' flanking regions are included in SEQ ID NO:9 and given in SEQ ID NOs:21 and 22.

The junction sequences are relatively short polynucleotide molecules that are novel DNA sequences and are diagnostic for MON89788 DNA when detected in a polynucleic acid detection assay. The junction sequences in SEQ ID NO:1 and SEQ ID NO:2 represent 10 polynucleotides on each side of an insertion site of the transgene fragment and soybean genomic DNA in MON89788. Longer or shorter polynucleotide junction sequences can be selected from SEQ ID NO:3 or SEQ ID NO:4. The junction molecules (5'junction region SEQ ID NO:1, and 3' junction region SEQ ID NO:2) are useful as DNA probes or as DNA primer molecules in methods for DNA detection.

Primers and probes used in a Taqman® method (Roche Molecular Systems, Inc., Pleasanton, CA) for detection of an event specific DNA molecule were developed for event MON89788. The primer molecules are referred to as SQ2824 (SEQ ID NO:10), SQ2826 (SEQ ID NO:11), SQ1141 (SEQ ID NO:12), SQ1142 (SEQ ID NO:13), SQ5543 (SEQ ID NO:14) and the probe molecules are referred to as PB871-6FAM (SEQ ID NO:15), PB2191-VIC (SEQ ID NO:16), and PB57-VIC (SEQ ID NO:17). The primers and probes were used in the Taqman® method according to manufacturers instructions to provide a diagnostic amplicon for DNA comprising MON89788. Soybean tissues including processed products, for example meal, are useful sources of DNA for this method. Additional primers used to produce an amplicon from soymeal include SEQ ID NOs:18-20.

Table 3. Genome and genetic element annotation of the transgene/genomic DNA fragment (SEQ ID NO:9) contained in the genome of soybean comprising MON89788.

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Sequence flanking 5' end of the insert	1-1103	***SOYBEAN GENOMIC DNA***
5'Junction region	1093-1113	DNA region spanning the transgene insertion
B[3]–Right Border	1104-1145	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al.*, 1982)

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Intervening Sequence	1146-1215	Sequences used in DNA cloning
P[4]–*FMV/Tsf1*	1216-2255	Chimeric promoter consisting of enhancer sequences from the 35S promoter of the Figwort Mosaic virus (Richins et al., 1987) and the promoter from the *Tsf1* gene of *Arabidopsis thaliana* (encoding elongation factor EF-1alpha (Axelos, *et al.*, 1989)
L[5]–*Tsf1*	2256-2301	5' nontranslated leader (exon 1) from the *Tsf1* gene of *Arabidopsis thaliana* encoding elongation factor EF-1 alpha (Axelos *et al.*, 1989)
I[6]–*Tsf1*	2302-2923	Intron from the *Tsf1* gene of *Arabidopsis thaliana* encoding elongation factor EF-1 alpha (Axelos *et al.*, 1989)
Intervening Sequence	2924-2932	*SEQUENCES USED IN DNA CLONING*
TS[7]–*CTP2*	2933-3160	Sequences encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee *et al.*, 1987)
CS[8]–*cp4 epsps*	3161-4528	Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Padgette *et al.*, 1996; Barry *et al.*, 1997)
Intervening Sequence	4529-4570	Sequences used in DNA cloning
T[9]–E9	4571-5213	3' nontranslated sequence from the *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit (Coruzzi *et al.*, 1984)
Intervening Sequence	5214-5256	Sequences used in DNA cloning
B–Left Border	5257-5406	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al.*, 1983)
3' junction region	5396-5416	DNA region spanning the transgene insertion
Sequence flanking 3' end of the insert	5407-6466	Soybean genomic DNA

Southern Blot Analysis

Genomic DNA from a plant comprising MON89788 and control soybean genomic DNA (~15μg of each) is digested with various restriction enzymes (140U) in a total volume of 150μl including 15μl of the corresponding manufacturer's buffer (NEB, Beverly, MA). Restriction endonucleases, *e.g.*, Bgl11, BamH1, Nco1, Hind111, and Bcl1, are used in the Southern analysis of MON89788. Endonuclease digests are performed at the appropriate temperature for at least 6 hours. After incubating, the DNA is precipitated with 3M sodium acetate and 2.5 volumes of ethanol. Subsequently, the DNA is washed with 70% ethanol, dried, and resuspended in 40μl of TBE. Loading buffer (0.2X) is added to the samples and then subjected to electrophoresis on agarose

gels (0.8%) for 16-18 hours at 30 volts. The gels are stained with ethidium-bromide, then treated with a depurination solution (0.125N HCL) for 10 minutes, with a denaturing solution (0.5M sodium hydroxide, 1.5M sodium chloride) for 30 minutes, and finally with a neutralizing solution (0.5M Trizma base, 1.5M sodium chloride) for 30 minutes. The DNA is transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Buckingamshire, England) using a Turboblotter (Schleicher and Schuell, Dassel, Germany) for 4-6 hours and then fixed to the membrane using a UV light.

Membranes are prehybridized with 20mls of DIG Easy Hyb solution (Roche Molecular Biochemicals, Indianapolis, IN; cat. #1603558) for 2-4 hours at 45°C. Radioactive DNA probes (^{32}P dCTP) homologous or complementary to SEQ ID NO:1, or SEQ ID NO:2, or SEQ ID NO:3, or SEQ ID NO:4, or a portion thereof are made using a Radprime DNA Labeling kit (Invitrogen, Carlsbad, CA; cat. #18428-011). Unincorporated nucleotides are removed using SEPHADEX G-50 columns (Invitrogen). The prehybridization solution is replaced with 10mls of pre-warmed DIG Easy Hyb solution containing the denatured probe to a final concentration of 1 million counts per ml. The blots are hybridized at 45°C for 16-18 hours.

Blots are washed with a low stringency solution (5X SSC, 0.1X SDS) at 45°C and then repeatedly washed with a higher stringency solution (0.1X SSC, 0.1% SDS) at 65°C. The blots are exposed to a phosphor screen (Amersham Biosciences, Piscataway, NJ) for >2 hours and the exposure read using a Data Storm 860 machine (Amersham Biosciences). These methods and conditions exemplified may be modified by those skilled in the art of detecting DNA in a sample.

EXAMPLE 3

Weed Control

Controlling the growth of weeds in a field of soybeans comprising MON89788. A field is planted with soybean seeds comprising MON89788, the seeds are allowed to germinate into plants and the field of plants is treated with a herbicide formulation containing glyphosate. An effective dose of a glyphosate formulation at treatment rates of from about 0.25 lb ae/A (pounds of glyphosate acid equivalent/acre) to 3 or more lb ae/A is applied to the field. Rates often applied range from about 0.75 lb ae/A to 1.5 lb ae/A at a frequency of one or more treatments during the growing season as necessary to control

the growth of weeds in a field. Seeds from the plants comprising MON89788 are harvested from the treated plants.

A deposit of the Monsanto Technology LLC, soybean seed representative of event MON89788 disclosed above and recited in the claims has been made under the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110. The ATCC accession number for the deposit comprising event MON89788 (also known as MON19788 or GM_A19788) is PTA-6708, deposited May 11, 2005. The deposit will be maintained in the depository for a period of 30 years, or 5 years after the last request, or for the effective life of the patent, whichever is longer, and will be replaced as necessary during that period.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS:

1. A nucleic acid sequence comprising the sequence of SEQ ID NO:1 or SEQ ID NO:2.

2. A soybean plant or part thereof comprising event MON89788, wherein representative soybean seed comprising event MON89788 have been deposited under ATCC accession number PTA-6708.

3. A seed of the plant of claim 2, wherein the seed comprises event MON89788.

4. A soybean commodity product produced from the seed of claim 3.

5. The soybean commodity product of claim 5, further defined as meal, flour, flakes, or oil.

6. The soybean plant part of claim 2, defined as a cell, pollen, ovule, flower, shoot, root, or leaf.

7. The soybean plant of claim 2, further defined as a progeny plant of any generation of a soybean plant comprising said event MON89788.

8. The soybean plant of claim 2, wherein the genome of said plant comprises at least one DNA molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:21 and SEQ ID NO:22.

9. The soybean plant of claim 2, wherein the genome of said plant produces an amplicon diagnostic for event MON89788 when tested in a DNA amplification method, said amplicon comprising SEQ ID NO:1 or SEQ ID NO:2.

10. The seed of claim 3, wherein the DNA of the seed produces an amplicon diagnostic for event MON89788 when tested in a DNA amplification method, said amplicon comprising SEQ ID NO:1 or SEQ ID NO:2.

11. The meal, flour, flakes, or oil of claim 5, further defined as comprising a nucleic acid that produces an amplicon diagnostic for event MON89788 when tested in a DNA amplification method, said amplicon comprising SEQ ID NO:1 or SEQ ID NO:2.

12. A DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:3, or its complement that is useful in a DNA amplification method to produce an amplicon diagnostic for event MON89788.

13. An isolated DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:4, or its complement that is useful in a DNA amplification method to produce an amplicon diagnostic for event MON89788.

14. A DNA detection kit specific for event MON89788 comprising at least nucleic acid comprising 11 or more contiguous nucleotides homologous or complementary to SEQ ID NO:3 or SEQ ID NO:4.

15. A method of producing a soybean plant tolerant to glyphosate herbicide comprising introducing into the genome of said plant event MON89788.

16. The method of claim 15, defined as comprising the steps of :

(a) crossing a first soybean plant comprising event MON89788 with a second soybean plant lacking event MON89788 to produce progeny plants; and

(b) selecting at least a first progeny plant that comprises said event MON89788 and is tolerant to glyphosate.

17. The method of claim 16, further comprising selfing said first progeny plant to produce second generation progeny plants and selecting at least a first plant homozygous for said event MON89788.

18. A method of detecting the presence of DNA corresponding to the soybean event MON89788 in a sample, the method comprising:

(a) contacting a sample comprising soybean DNA with a primer set, which when used in a nucleic acid amplification reaction with genomic DNA from soybean event MON89788, produces a diagnostic amplicon for soybean event MON89788; and

(b) performing a nucleic acid amplification reaction, thereby producing the diagnostic amplicon; and

(c) detecting the diagnostic amplicon.

19. A method of detecting the presence of a nucleic acid corresponding to event MON89788 in a sample, the method comprising:

(a) obtaining a sample of soybean DNA; and

(b) assaying the sample for the presence of a DNA sequence from event MON89788.

20. The method of claim 19, wherein assaying the DNA sample comprises detecting the presence of the nucleic acid sequence of at least one of SEQ ID NO:1, SEQ ID NO:2, or complements thereof.

21. A soybean plant comprising a glyphosate tolerant trait that is genetically linked to a nucleic acid molecule comprising SEQ ID NO:1 or SEQ ID NO:2.

22. A method of producing a soybean commodity product comprising,

(a) obtaining the soybean plant or part thereof of claim 2; and

(b) producing a soybean commodity product from the soybean plant or part thereof.

23. The method of claim 22, wherein the commodity product is defined as meal, flour, flakes, protein isolate, or oil.

24. A method for controlling the growth of weeds in a field comprising soybean plants comprising event MON89788, the method comprising treating the field with an amount of glyphosate effective to control the growth of weeds, wherein the soybean plants exhibit tolerance to the glyphosate.

25. The method of claim 24, wherein treating the field is carried out from V1 to R4 stage of growth.

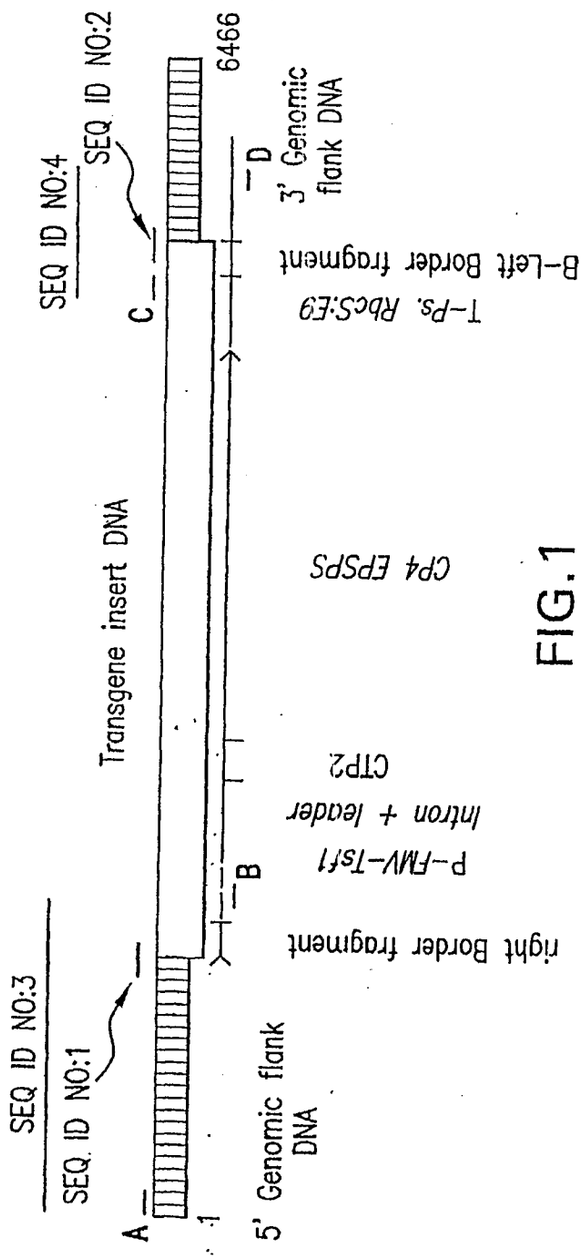

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FIG. 1

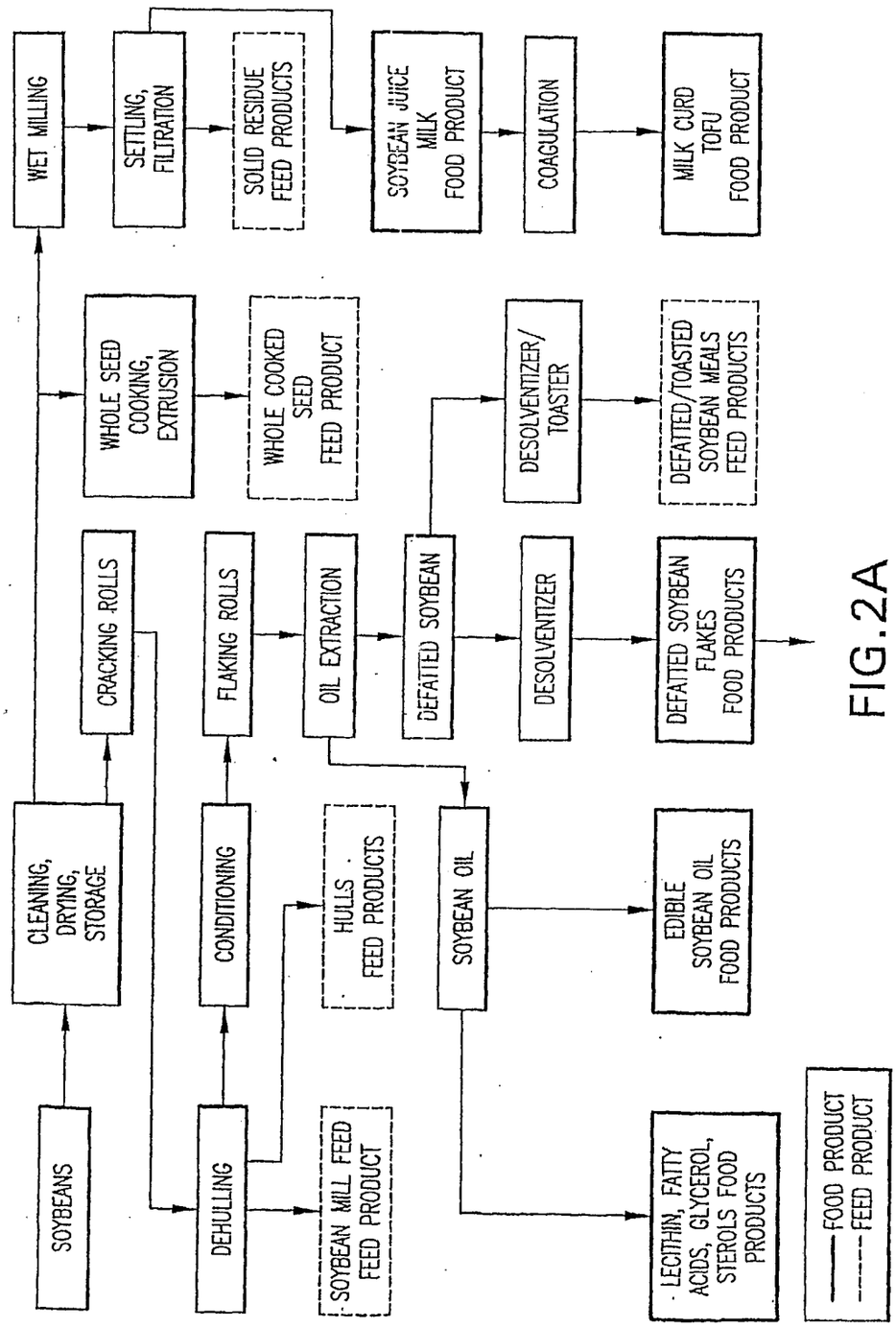

WET MILLING
SETTLING, FILTRATION
SOLID RESIDUE FEED PRODUCTS
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COAGULATION
MILK CURD TOFU FOOD PRODUCT
WHOLE SEED COOKING, EXTRUSION
WHOLE COOKED SEED FEED PRODUCT
DESOLVENTIZER/ TOASTER
DEFATTED/TOASTED SOYBEAN MEALS FEED PRODUCTS
CRACKING ROLLS
FLAKING ROLLS
OIL EXTRACTION
DEFATTED SOYBEAN
DESOLVENTIZER
DEFATTED SOYBEAN FLAKES FOOD PRODUCTS
CLEANING, DRYING, STORAGE
CONDITIONING
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SOYBEAN OIL
EDIBLE SOYBEAN OIL FOOD PRODUCTS
SOYBEANS
DEHULLING
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FOOD PRODUCT
FEED PRODUCT

FIG.2A

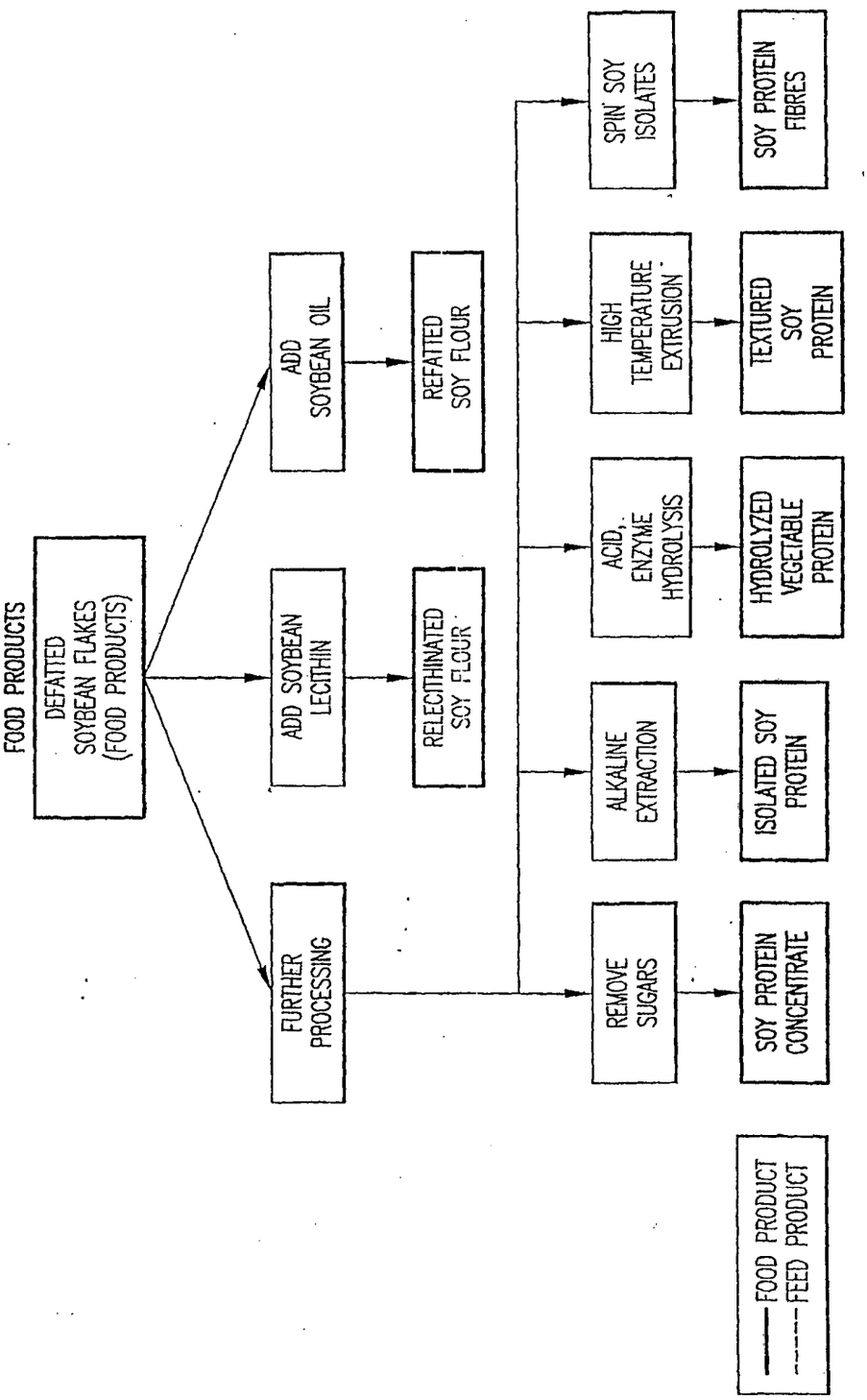

FOOD PRODUCTS
DEFATTED SOYBEAN FLAKES (FOOD PRODUCTS)
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ADD SOYBEAN LECITHIN
FURTHER PROCESSING
REFATTED SOY FLOUR
RELECITHINATED SOY FLOUR
REMOVE SUGARS
ALKALINE EXTRACTION
ACID, ENZYME HYDROLYSIS
HIGH TEMPERATURE EXTRUSION
SPIN SOY ISOLATES
SOY PROTEIN CONCENTRATE
ISOLATED SOY PROTEIN
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FIG.2B

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(71) **Solicitante** *(para todos los estados designados excepto US)*: **MONSANTO TECHNOLOGY LLC** [--/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US).

(72) **Inventores; e**

(75) **Inventores/Solicitantes** *(solo para US)*: **MALVEN, Marianne** [--/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US). **RINEHART, Jennifer** [--/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US). **TAYLOR, Nancy** [--/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US). **DICKINSON, Ellen** [--/US]; 800 North Lindberg Blvd., St. Louis, MO 63167 (US).

(74) **Agente: HANSON, Robert, E.**; FULBRIGHT & JAWORSKI L.L.P., 600 Congress Avenue, Suite 2400, Austin, TX 78701 (US).

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(54) Título: EVENTO MON89788 DE SOYA Y METODOS PARA LA DETECCION DEL MISMO

(57) Resumen: La presente invención proporciona un evento de transformación MON89788 para plantas de frijol de soya y semillas y moléculas de ADN únicas a estos eventos. La invención también proporciona métodos para detectar la presencia de estas moléculas de ADN en una muestra.

EVENTO MON89788 DE SOYA Y METODOS PARA LA DETECCION DEL MISMO

REFERENCIA CRUZADA A SOLICITUDES RELACIONADAS

Esta solicitud reclama prioridad de la Solicitud Provisional U.S. No. 60/685,584, presentada el 27 de mayo de 2005, cuya descripción total es incorporada por tanto para referencia.

CAMPO DE LA INVENCION

La presente invención se refiere a un evento de transformación de soya transgénica novedoso y distintivo, designado como MON89788, un cultivo de soya derivado de éste, y partes vegetales, semillas y productos del mismo. La invención también se refiere a ensayos para detectar la presencia de una molécula de ADN específica para MON89788 en un extracto de parte de la planta o extracto de la semilla.

ANTECEDENTES DE LA INVENCION

La soya (*Glycine max*) es un cultivo importante en muchas áreas del mundo. Los métodos de biotecnología se han aplicado a la soya para el mejoramiento de las características agronómicas y la calidad del producto.

Una de dicha característica agronómica importante en la producción de soya es la tolerancia a herbicida, en particular, tolerancia al herbicida de glifosato. Un evento de soya tolerante a herbicida puede ser una característica útil para el manejo de malezas.

N-fosfonometilglicina, también conocida como glifosato, es un herbicida bien conocido que tiene actividad en amplio espectro de especies vegetales. Glifosato es el ingrediente activo de Roundup® (Monsanto Co., St. Louis, MO), un herbicida seguro que tiene una vida media deseablemente corta en el ambiente. Cuando se aplica a una superficie vegetal, el glifosato se mueve sistémicamente a través de la planta. Glifosato es fitotóxico debido a su inhibición de la trayectoria de ácido shikímico, que provee un precursor para la síntesis de aminoácidos aromáticos. El glifosato inhibe la enzima 5-enolpiruvil-3-fosfoshikimato sintasa (EPSPS) encontrada en las plantas.

La tolerancia a glifosato se puede lograr por medio de la expresión de variantes de EPSPS que tienen afinidad más baja para glifosato y por tanto retienen su actividad catalítica en presencia de glifosato (Patente de E.U.A. Nos. 5,633,435; 5,094,945; 4,535,060 y 6,040,497). Las enzimas que degradan glifosato en tejidos vegetales (Patente de E.U.A. No. 5,463,175) también tienen la capacidad de conferir tolerancia celular a glifosato. Dichos genes se usan para la producción de cultivos transgénicos que sean tolerantes a glifosato, permitiendo de este modo que se use glifosato para el control efectivo de la maleza con una mínima consecuencia en el daño del cultivo. Por ejemplo, la tolerancia a glifosato se ha diseñado genéticamente en

maíz (Patente de E.U.A. No. 5,554,798), trigo (Patente de E.U.A. No. 6,689,880), algodón (Patente de E.U.A. No. 6,740,488), soya (WO 9200377) y canola (Solicitud de Patente US 20040018518). Los transgenes para tolerancia a glifosato y los transgenes para tolerancia a otros herbicidas, por ejemplo, el gen bar (Toki et al., 1992; Thompson et al., 1987; fosfinotricina acetiltransferasa (DeBlock et al., 1987), para tolerancia a herbicida de glufosinato) también son útiles como marcadores que se pueden seleccionar o marcadores que se pueden marcar y pueden proveer un fenotipo útil para la selección de plantas enlazadas con otras características agronómicamente útiles.

La expresión de genes extraños en plantas se sabe que es influenciada por su posición cromosomal, probablemente debido a la estructura de cromatina (por ejemplo, heterocromatina) o la proximidad de elementos de regulación transcripcional (por ejemplo, potenciadores) cerca del sitio de integración (Weising et al., 1988). Por esta razón, a veces es necesario seleccionar un número grande de eventos con el fin de identificar un evento caracterizado por expresión óptima de un gen introducido de interés. Por ejemplo, se ha observado en plantas y en otros organismos que puede existir una variación amplia en niveles de expresión de un gen introducido entre eventos. También pueden existir diferencias en patrones espaciales o temporales de expresión, por ejemplo, diferencias en la expresión relativa de un transgen en varios tejidos vegetales, que no pueden corresponder a los patrones esperados de los elementos reguladores transcripcionales presentes

en la construcción génica introducida. Por esta razón, es común producir cientos a miles de diferentes eventos y seleccionar aquellos eventos para un evento sencillo que tiene niveles de expresión transgénica deseados y patrones para propósitos comerciales. Un evento que tiene niveles deseados o patrones deseados de expresión transgénica es útil para introgresión del transgen en otros fondos genéticos por medio de cruzamiento de distinto tipo pero de la misma raza sexual usando métodos convencionales de reproducción. La progenie de dichas cruzas mantiene las características de expresión transgénica del transformante original. Esta estrategia se usa para asegurar una expresión génica confiable en un número de variedades que se adaptan bien a condiciones locales de crecimiento.

Podría ser conveniente tener la capacidad de detectar la presencia de un evento particular con el fin de determinar si la progenie de una cruza sexual contiene un transgen de interés. Además, un método para detectar un evento particular podría ser útil para cumplir con las regulaciones que requieren la aprobación pre-mercado y etiquetado de alimentos derivados de plantas de cultivo recombinante, por ejemplo. Es posible detectar la presencia de un transgen por cualquier método de detección de ácido polinucleico bien conocido tal como la reacción de cadena polimerasa (PCR) o hibridación de ADN usando sondas de ácido polinucleico. Estos métodos de detección generalmente se enfocan en los elementos genéticos frecuentemente usados, tales como promotores, terminadores, genes marcadores, etc. Como un resultado, dichos métodos no pueden ser útiles

para discriminar entre diferentes eventos, particularmente aquellos producidos usando la misma construcción de ADN a menos que la secuencia de ADN cromosomal ("ADN de flanqueo") adyacente al ADN transgénico insertado se conozca. Se discute un ensayo de PCR específico del evento, por ejemplo, por Windels et al. (1999), que identificó el evento 40-3-2 de soya tolerante a glifosato por PCR usando un conjunto de cebadores que expanden la unión entre el transgen inserto y ADN de flanqueo, específicamente un cebador que incluye una secuencia desde el inserto y un segundo cebador que incluye una secuencia del ADN de flanqueo. Los métodos de detección de ADN específico del evento de planta transgénica se han descrito también en la Patente de E.U.A. Nos. 6,893,826; 6,825,400; 6,740,488; 6,733,974 y 6,689,880; 6,900,014 y 6,818,807, incorporadas aquí para referencia de manera íntegra.

Esta invención se refiere al evento MON89788 de soya tolerante a glifosato (también referido como MON19788 o GM_A19788) y a las moléculas de ADN contenidas en estas plantas de soya que son útiles en los métodos de detección para la planta y su progenie y tejidos de planta derivados del MON89788.

BREVE DESCRIPCION DE LA INVENCION

La presente invención provee un evento transgénico de soya designado como MON89788 (también referido como MON19788) y su progenie que tiene semilla representativa depositada con Colección de

Cultivos Tipo Americana (ATCC) con No. de acceso No. PTA-6708. Otro aspecto de la invención son las células vegetales o partes regenerables de la planta y semillas del evento MON89788 de soya. La invención también incluye partes de plantas del evento, pero no se limita a una célula, polen, óvulo, flores, brotes, raíces, hojas, y productos derivados de MON89788, por ejemplo alimento, harina y aceite de soya.

Un aspecto de la invención provee composiciones y métodos para detectar la presencia de un ADN transgénico/región de unión genómica de un evento MON89788 de soya, planta o semilla o productos derivados de partes de plantas o semilla. Las moléculas de ADN se proveen comprendiendo al menos una molécula de ADN transgénico/de unión genómica seleccionada del grupo que consta de SEQ ID NO: 1 y SEQ ID NO: 2, y sus complementos, en donde la molécula de unión extiende el sitio de inserción que comprende un ADN heterólogo insertado en el genoma de la célula de soya y el ADN genómico de la célula de soya que flanquea el evento MON89788 de soya en sitio de inserción. Dichas secuencias de unión pueden, en un aspecto de la invención, definirse como comprendiendo nucleótidos 1093-1113 o 5396-5416 de SEQ ID NO: 9, respectivamente. En otros aspectos de la invención, las uniones se pueden definir como incluyendo porciones adicionales del genoma de flanqueo y transgen, por ejemplo, y se pueden definir como comprendiendo una o más secuencias según se proporciona por los nucleótidos 1073-1113, 1043-1113, 1093-1133, 1093-1163, 1043-1163, 5376-5416, 5346-5416, 5396-5436, 5396-5416, 5396-5466,

o 5346-5466 de SEQ ID NO: 9. Dichas secuencias y plantas y semillas que comprenden estas secuencias por tanto forman un aspecto de la invención.

Se provee una molécula de ADN novedosa que es un transgen de ADN/región genómica SEQ ID NO: 3 o su complemento, del evento MON89788 de soya. Una planta y semilla de soya que comprenden SEQ ID NO: 3 en su genoma es un aspecto de esta invención. SEQ ID NO: 3 comprende además SEQ ID NO: 1 de manera íntegra.

De acuerdo a otro aspecto de la invención, se provee una molécula de ADN que es un transgen de ADN/región genómica SEQ ID NO: 4, o su complemento, en donde esta molécula de ADN es novedosa en el evento MON89788 de soya. Una planta y semilla de soya que comprenden SEQ ID NO: 4 en su genoma es un aspecto de esta invención. SEQ ID NO: 4 comprende además SEQ ID NO: 2 de manera íntegra.

De acuerdo a otro aspecto de la invención, dos moléculas de ácido nucleico se proveen para usarse en un método de detección de ADN, en donde la primera molécula de ácido nucleico comprende al menos 11 o más polinucleótidos contiguos de cualquier porción de la región transgénica de la molécula de ADN de SEQ ID NO: 3 y el segundo ácido nucleico es una molécula de longitud similar de cualquier porción de una región de ADN genómico de soya de flanqueo 5' de SEQ ID NO: 3, en donde estas moléculas de ácido nucleico cuando se usan en conjunto son útiles como cebadores en un método de amplificación de ADN que produce un amplicón. El amplicón producido usando estos cebadores en el método de amplificación de ADN es

diagnóstico para el ADN del evento MON89788 de soya. El amplicón producido por los cebadores descritos que es homólogo o complementario a una porción de SEQ ID NO: 3 que comprende SEQ ID NO: 1 es un aspecto de la invención.

Se proveen de acuerdo a otro aspecto de la invención, dos moléculas de ácido nucleico para usarse en un método de detección de ADN, en donde la primera molécula de ácido nucleico comprende al menos 11 o más polinucleótidos contiguos de cualquier porción de la región transgénica de la molécula de ADN de SEQ ID NO: 4 y una segunda molécula de ácido nucleico de longitud similar de cualquier porción de ADN genómico de soya de flanqueo 3' de SEQ ID NO: 4, en donde estas moléculas de ácido nucleico cuando se usan en conjunto son útiles como cebadores en un método de amplificación de ADN que produce un amplicón. El amplicón producido usando estos cebadores en el método de amplificación de ADN es diagnóstico para el ADN del evento MON89788. El amplicón producido por los cebadores descritos que es homólogo o complementario a una porción de SEQ ID NO: 4 que comprende SEQ ID NO: 2 es un aspecto de la invención.

Cualquier par cebador de ácido nucleico derivado de SEQ ID NO: 3 o SEQ ID NO: 4, o SEQ ID NO: 9 o sus complementos, que cuando se usa en una reacción de amplificación de ADN produce un amplicón diagóstico para el tejido derivado del evento MON89788 de soya, tal como un amplicón que comprende SEQ ID NO: 1 o SEQ ID NO: 2 o cualquier porción de SEQ ID NO: 9, respectivamente, es otra modalidad de la invención. En una modalidad

particular, el par cebador puede consistir de cebador A (SEQ ID NO: 5) y cebador D (SEQ ID NO: 8).

Otro aspecto de la invención es una planta de soya, o semilla o producto derivado de una planta o semilla que comprende el evento MON89788, en donde el ADN genómico cuando se aísla de la planta de soya, o semilla, o producto produce un amplicón en un método de amplificación de ADN que comprende SEQ ID NO: 1 o SEQ ID NO: 2.

Otro aspecto todavía de la invención es una planta de soya, o semilla, o producto derivado de una planta o semilla que comprende MON89788, en donde el ADN genómico cuando se aísla de la planta de soya, o semilla, o producto produce un amplicón en un método de amplificación de ADN, en donde las moléculas de cebador de ADN SEQ ID NO: 5 y SEQ ID NO: 6 se usan en el método de amplificación de ADN.

Aún otro aspecto de la invención es una planta de soya, semilla, producto, o mercancía derivada de la planta o semilla, que comprende MON89788, en donde el ADN genómico cuando se aísla de la planta de soya, o semilla, o producto produce un amplicón en un método de amplificación de ADN, en donde las moléculas de cebador de ADN SEQ ID NO: 7 y SEQ ID NO: 8 se usan en el método de amplificación de ADN. El producto o mercancía puede comprender, sin limitación, un alimento o producto alimenticio que comprende o se deriva de uno o más de los siguientes productos de una planta de soya que comprende el evento MON89788: lecitina, ácidos grasos, glicerol, esterol, aceite comestible, hojuelas de soya

desengrasada, alimentos de soya incluyendo alimentos de soya desengrasados y tostados, leche de soya cuajada, tofu, harina de soya, concentrado de proteína de soya, proteína de soya aislada, proteína vegetal hidrolizada, proteína de soya texturizada, y fibra de proteína de soya.

De acuerdo con otro aspecto de la invención, un método para detectar la presencia de ADN correspondiente específicamente al ADN del evento MON89788 de soya en una muestra se proporciona. Dicho método comprendiendo: (a) poner en contacto una muestra que comprende ADN con un par cebador de ADN; (b) realizar una reacción de amplificación de ácido nucleico, produciendo de este modo el amplicón, y (c) detectar el amplicón, en donde dicho amplicón comprende SEQ ID NO: 1 o SEQ ID NO: 2. Un equipo que comprende moléculas cebadoras de ADN que cuando se usa en un método de amplificación de ADN produce un amplicón que comprende SEQ ID NO: 1 o SEQ ID NO: 2 es un aspecto adicional de la invención.

De acuerdo a otro aspecto de la invención, un método para detectar la presencia de ADN que corresponde específicamente al ADN del evento MON89788 de soya en una muestra se proporciona. Dicho método comprendiendo: (a) poner en contacto una muestra que comprende ADN con una sonda que hibrida bajo condiciones de hibridación rigurosas con ADN genómico del evento MON89788 de soya y no hibrida bajo las condiciones de hibridación rigurosas con un ADN de planta de soya de control; (b) someter la muestra y la sonda a condiciones de hibridación rigurosas; y (c) detectar la hibridación de la sonda al ADN del evento MON89788 de soya, en donde

dicha sonda comprende SEQ ID NO: 1 o SEQ ID NO: 2. La muestra puede comprender una semilla de progenie, planta, o parte de la planta que comprende el evento MON89788 de soya, o cualquiera de los siguientes productos derivados de una planta que comprende MON89788: lecitina, ácidos grasos, glicerol, esterol, aceite comestible, hojuelas de soya desengrasada, alimentos de soya incluyendo alimentos de soya desengrasados y tostados, leche de soya cuajada, tofu, harina de soya, concentrado de proteína de soya, proteína de soya aislada, proteína vegetal hidrolizada, proteína de soya texturizada, y fibra de proteína de soya. Un equipo que comprende una sonda de ADN que comprende una molécula de ADN que es homóloga o complementaria a SEQ ID NO: 1 o SEQ ID NO: 2, es un aspecto de la invención. Un equipo que comprende una molécula de ADN que comprende SEQ ID NO: 18, SEQ ID NO: 19 o SEQ ID NO: 20, o sus complementos, también es un aspecto de la invención.

De acuerdo a otro aspecto de la invención, se proporciona un método para producir una planta de soya que tolera una aplicación de glifosato que comprende las etapas de: (a) cruzar sexualmente una primera planta de soya tolerante a glifosato parental que comprende el evento MON89788, y una segunda planta de soya parental que carece de tolerancia a glifosato, produciendo de este modo una pluralidad de plantas de progenie; y (b) seleccionar una planta de progenie que tolera la aplicación de glifosato. Los métodos de reproducción pueden comprender adicionalmente las etapas de cruzar la planta parental que comprende el evento MON89788 de soya a

una segunda planta de soya parental que también es tolerante a glifosato y seleccionar la progenie tolerante a glifosato por medio de ADN marcador molecular enlazado de manera genética al fenotipo tolerante a glifostao encontrado en cada madre.

Otro aspecto de la invención es un método para controlar las malezas en un campo de plantas de soya que comprenden MON89788, en donde dicho método comprende la plantación de un campo con semilla de soya que comprende el evento MON89788 dicha semilla representativa depositada como No de acceso ATCC PTA-6708, permitiendo que dicha semilla germine y el tratamiento de dichas plantas con una dosis efectiva de glifosato para controlar el crecimiento de la maleza en dicho campo.

Los aspectos anteriores y otros de la invención serán más aparentes de la siguiente descripción detallada y dibujos anexos.

BREVE DESCRIPCION DE LOS DIBUJOS

La figura 1 muestra la organización de la inserción transgénica en el genoma de una planta de soya que comprende el evento MON89788.

Las figuras 2A-2B muestran el procesamiento de productos de consumo de soya.

61

DESCRIPCION DETALLADA DE LA INVENCION

La presente invención se refiere a un evento de transformación de soya novedoso designado como MON89788 que provee tolerancia a glifosato, y las partes de la planta y semilla y productos producidos a partir de las plantas, partes de la planta, semillas, y productos que comprenden el evento. La invención provee moléculas de ADN que son novedosas en el genoma de células de soya que comprenden MON89788 y moléculas de ADN que se pueden usar en varios métodos de detección de ADN para identificar el ADN de MON89788 en una muestra. La invención provee un método para controlar malezas en un campo de plantas que contienen MON89788 al tratar las malezas en el campo que comprende plantas que comprenden el evento MON89788 con un herbicida glifosato.

Las siguientes definiciones y métodos se proporcionan para definir mejor la presente invención y guiar a aquellos con experiencia en la técnica en la práctica de la presente invención. A menos que se indique lo contrario, los términos son para ser entendidos de acuerdo al uso convencional para aquellos con experiencia en la técnica relevante. Las definiciones de términos comunes en la biología molecular también se pueden encontrar en Rieger et al. (1991) y Lewin (1994). La nomenclatura para bases de ADN se exponen en 37CFR§ 1.822, se usa.

Como se usa en la presente, el término "soya" se refiere a glicina max e incluye todas las variedades de la planta que se pueden procrear con

62

soya.

Como se usa aquí, el término "que comprende" se refiere a "incluyendo pero sin limitarse a".

"Glifosato" se refiere a N-fosfonometilglicina y sus sales, glifosato es el ingrediente activo de herbicida Roundup® (Monsanto Co.). Tratamientos con "herbicida glifosato" se refieren a tratamientos con el herbicida Roundup®, Roundup Ultra®, Roundup Pro® o cualquier otra formulación de herbicida que contiene glifosato. Ejemplos de formulaciones comerciales de glifosato incluyen, sin restricción, aquellas comercializadas por Monsanto Company como herbicidas ROUNDUP®, ROUNDUP® ULTRA, ROUNDUP® ULTRAMAX, ROUNDUP® CT, ROUNDUP® EXTRA, ROUNDUP® BIACTIVE, ROUNDUP® BIOFORCE, RODEO®, POLARIS®, SPARK® y ACCORD®, todos lo cuales contienen glifosato como sus sal isopropilamonio; ROUNDUP®, WEATHERMAX (sal de potasio glifosato), aquellos comercializadas por Monsanto Company como herbicidas ROUNDUP® DRY y RIVAL®, que contienen glifosato como su sal de amonio; que se comercializa por Monsanto Company como ROUNDUP® GEOFORCE, que contiene glifosato como su sal de sodio; y que se comercializa por Syngenta Crop Protection como el herbicida TOUCHDOWN®, que contiene glifosato como su sal trimetilsulfonio. El tratamiento de un campo que comprende plantas de soya tolerantes a glifosato que comprenden el evento MON89788 con cualquiera de estas formulaciones de herbicida de glifosato controlaran el crecimiento de la maleza en el campo y no afectan el crecimiento o

rendimiento de las plantas de soya que comprenden MON89788.

Un "evento" transgénico se produce por la transformación de células vegetales con ADN heterólogo, por ejemplo, una construcción de ácido nucleico que incluye un transgen de interés, regeneración de una población de plantas que resulta de la inserción del transgen en el genoma de la planta, y selección de una planta particular caracterizada por medio de la inserción en un sitio de genoma particular. El término "evento" se refiere al transformante original y progenie del transformante que incluye el ADN heterólogo. El término "evento" también se refiere a la progenie producida por un cruzamiento sexual entre el transformante y otra variedad que incluye el ADN transgénico heterólogo y el ADN genómico de flanqueo. El término "evento" también se refiere al ADN del transformante original que comprende el ADN insertado y secuencia genómica de flanqueo inmediatamente adyacente al ADN insertado que podría esperarse se transfiera a una progenie que recibe ADN insertado incluyendo el transgen de interés como el resultado de un cruce sexual de una línea parental que incluye el ADN insertado (por ejemplo, el transformante original y progenie que resultan de auto-polinización)y una línea parental que no contiene el ADN insertado.

Una planta de soya tolerante a glifosato se puede procrear por medio de un primer cruce sexualmente de una primera planta de soya parental que consta de una planta de soya que se desarrolla de una planta de soya tolerante a glifosato transgénica que comprende MON89788 o una planta de soya que es una progenie de la cruza de dicha planta que expresa

el fenotipo tolerante a glifosato, y una segunda planta de soya parental que carece de tolerancia a glifosato, produciendo de este modo una pluralidad de primeras plantas de progenie; y después la selección de una planta de progenie que es tolerante a la aplicación de herbicida de glifosato. Estas etapas pueden incluir además el retro-cruzamiento de la planta de progenie tolerante a glifosato a la segunda planta de soya parental o una tercera planta de soya parental, después la selección de la progenie mediante la aplicación con glifosato o por medio de identificación con marcadores moleculares asociados con la característica produciendo de este modo una planta de soya que tolera la aplicación de herbicida glifosato. Los marcadores moleculares se pueden usar comprendiendo las moléculas de ADN de unión identificadas en los sitios 5' y 3' de inserción del transgen en el evento MON89788.

También se entiende que dos diferentes plantas transgénicas se pueden aparear también para producir descendencia que contiene dos transgenes exógenos de segregación independientemente. El retro-cruce a una planta parental y un cruzamiento de distinto tipo pero de la misma raza con una planta no transgénica como se describió previamente también se contempla, como es la propagación vegetativa. Descripciones de otros métodos de procreación que son comúnmente usados para diferentes características y cultivos se pueden encontrar en una de las varias referencias, por ejemplo, Fehr, (1987).

Una "sonda" es un ácido nucleico aislado al cual se une una etiqueta detectable convencional o molécula reportera, por ejemplo, un

67

isótopo radioactivo, un ligando, un agente quimio-luminiscente, o una enzima. Dicha sonda es complementaria a una cadena de un ácido nucleico objetivo, en el caso de la presente invención, a una cadena de ADN genómico de una planta de soya que comprende el evento MON89788 ya sea de una planta de soya o semilla o de una muestra o extracto de la planta o semilla que incluye ADN del evento. Las sondas de acuerdo a la presente invención incluyen no solo ácidos desoxirribonucleico o ribonucleico, sino también poliamidas y otros materiales de sonda que se unen específicamente a un a secuencia de ADN objetivo y se pueden usar para detectar la presencia de aquella secuencia de ADN objetivo.

"Cebadores" son ácidos polinucleicos aislados que son fijados a una cadena de ácido polinucleico objetivo complementario por medio de hibridación de ácido nucleico para formar un híbrido entre el cebador y la cadena de ácido polinucleico objetivo, y después se extiende a lo largo de la cadena de ácido polinucleico objetivo por medio de polimerasa, por ejemplo, una ADN polimerasa. Los pares de cebador de la presente invención se refieren a su uso para la amplificación de una molécula de ácido polinucleico objetivo, por ejemplo, por medio de la reacción en cadena de polimerasa (PCR) u otros métodos de amplificación de ácido nucleico convencionales.

Las sondas y cebadores generalmente son 11 polinucleótidos o más en longitud, preferiblemente 18 polinucleótidos o más, más preferiblemente 24 polinucleótidos o 30 polinucleótidos o más. Dichas sondas y cebadores hibridan específicamente a una molécula objetivo bajo

condiciones de hibridación muy rigurosas. Preferiblemente, las sondas y cebadores de acuerdo a la presente invención tienen identidad de secuencia completa con la molécula objetivo, aunque las sondas que difieren de las condiciones severas y que retienen la capacidad para hibridar a secuencias objetivo bajo condiciones muy rigurosas se pueden diseñar por medio de métodos convencionales.

Los métodos para la preparación y uso de las sondas y cebadores se describen, por ejemplo, en Sambrook et al. (1989); Ausubel et al. (1992); e Innis et al. (1990). Los pares PCR-cebador (un conjunto de cebadores) se pude derivar de una secuencia conocida, por ejemplo, al usar programas de computadora destinados para aquel propósito tal como cebador (Versión 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Los cebadores y sondas basados en el ADN genómico de flanqueo y secuencias de inserto descritos aquí (SEQ ID NOs: 1-4 y 9) se pueden usar para confirmar y, si es necesario, corregir las secuencias descritas por métodos convencionales, por ejemplo, al aislar la molécula de ADN correspondiente de un depósito de semilla que comprende MON89788, y determinar la secuencia de ácido nucleico de dichas moléculas. Las moléculas de ADN asociadas adicionales se pueden aislar del genoma de una célula que comprende MON89788 que comprende el inserto transgénico y regiones de flanqueo genómicas, y fragmentos de estas moléculas se pueden usar como cebadores o sondas.

Las sondas y cebadores de ácido nucleico de la presente invención hibridan bajo condiciones rigurosas a una secuencia de ADN objetivo. Cualquier método de hibridación o amplificación de ácido nucleico convencional se puede usar para identificar la presencia de ADN del evento MON89788 en una muestra. Las moléculas de ácido nucleico o sus fragmentos tienen la capacidad de hibridar específicamente a otras moléculas de ácido nucleico bajo ciertas circunstancias. Como se usa aquí, se dice que dos moléculas de ácido nucleico son capaces de hibridar específicamente a otra si las dos moléculas tienen la capacidad de formar una estructura de ácido nucleico de doble cadena, anti-paralela y son de suficiente longitud para mantener esta estructura bajo condiciones muy rigurosas. Se dice que una molécula de ácido nucleico es el "complemento" de otra molécula de ácido nucleico si exhiben complementariedad completa. Como se usa en la presente, se dice que las moléculas exhiben "complementariedad completa" cuando cada nucleótido de una de las moléculas es complementaria a un nucleótido de la otra. Se dice que dos moléculas son "complementarias como mínimo" si pueden hibridar a otra con suficiente estabilidad para permitir que permanezcan fijas entre sí bajo al menos condiciones "poco rigurosas" convencionales. Similarmente, se dice que las moléculas son "complementarias" si pueden hibridar a otra con suficiente estabilidad para permitir que permanezcan fijas entre sí bajo condiciones "muy rigurosas" convencionales. Condiciones de rigurosidad convencional se describen por Sambrook et al., 1989, y por Haymes et al. (1985). Salidas de

complementariedad completa son por tanto permisibles, en tanto dichas salidas no eviten completamente la capacidad de las moléculas para formar una estructura de doble cadena. Con el fin de que una molécula de ácido nucleico sirva como un cebador o sonda necesita únicamente ser complementaria de manera suficiente en la secuencia para tener la capacidad de formar una estructura estable de doble cadena bajo el solvente particular y concentraciones de sal empleadas.

Como se usa en la presente, una secuencia homóloga sustancialmente es una secuencia de ácido nucleico que específicamente hibridará al complemento de la secuencia de ácido nucleico a la cual se está comparando bajo condiciones muy rigurosas. Condiciones de rigurosidad apropiada que promueven hibridación de ADN, por ejemplo, 6.0 x cloruro de sodio/ citrato de sodio (SSC) a aproximadamente 45°C, seguido por un lavado de 2.0 x SSC a 50°C, se conocen por aquellos con experiencia en la técnica o se pueden encontrar en Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Por ejemplo, la concentración de sal en la etapa de lavado se puede seleccionar de poco rigurosa de aproximadamente 2.0 x SSC a 50°C a muy rigurosa de aproximadamente 0.2 x SSC a 50°C. Además, la temperatura en la etapa de lavado se puede incrementar desde condiciones poco rigurosas a temperatura ambiente, aproximadamente 22°C, a condiciones muy rigurosas a aproximadamente 65°C. Ambas la temperatura y la sal pueden variar, o la temperatura o la concentración de la sal se pueden mantener constantes mientras la otra variable se cambia. En una modalidad

preferida, un ácido nucleico de la presente invención específicamente hibridará a una o más moléculas de ácido nucleico expuestas en SEQ ID NOs: 1-4, y 9, sus complementos o fragmentos de cualquiera bajo condiciones moderadamente rigurosas, por ejemplo a aproximadamente 2.0 x SSC y aproximadamente 65°C. En una modalidad particularmente preferida, un ácido nucleico de la presente invención hibridará específicamente a una o más de las moléculas de ácido nucleico expuestas en SEQ ID NOs: 1-4, y 9, sus complementos o fragmentos de cualquiera bajo condiciones muy rigurosas. En un aspecto de la presente invención, una molécula de ácido nucleico marcadora preferida de la presente invención comprende la secuencia de ácido nucleico como se expone en SEQ ID NO: 1 o SEQ ID NO: 2 o sus complementos o fragmentos de cualquiera. En otro aspecto de la presente invención, una molécula de ácido nucleico marcadora preferida de la presente invención comparte entre el 80% y 100% o 90% y 100% de identidad de secuencia con la secuencia de ácido nucleico expuesta en SEQ ID NO: 1 o SEQ ID NO: 2 o sus complementos o fragmentos de cualquiera. Moléculas de ADN marcadoras moleculares que comprenden SEQ ID NO: 1 o SEQ ID NO: 2, o sus complementos o fragmentos de cualquiera se puede usar como marcadores en métodos de procreación de plantas para identificar la progenie de cruces genéticos similares a los métodos descritos para análisis de marcador de ADN de repetición de secuencia simple, en Cregan et al. (1997); todas las cuales se incorporan aquí para referencia en su totalidad. La hibridación de la sonda a la molécula de ADN objetivo se puede detectar por

cualquier número de métodos conocidos por aquellos con experiencia en la técnica, estos pueden incluir, pero sin limitarse a: etiquetas fluorescentes, etiquetas radioactivas, etiquetas basadas anticuerpo, y etiquetas quimio-luminiscentes.

Con respecto a la amplificación de una secuencia de ácido nucleico objetivo (por ejemplo, por PCR) usando un par cebador de amplificación particular, "condiciones rigurosas" son condiciones que permiten que el par cebador hibridice únicamente a la secuencia ácido nucleico objetivo a la cual un cebador que tiene la secuencia tipo salvaje correspondiente (o su complemento) se puede unir y preferiblemente producir un producto de amplificación único, el amplicón, en una reacción de amplificación térmica de ADN.

El término "específico para (una secuencia objetivo)" indica que una sonda o cebador hibrida bajo condiciones de hibridación rigurosas solamente a la secuencia objetivo en una muestra que comprende la secuencia objetivo.

Como se usa en la presente, "ADN amplificado" o "amplicón" se refiere al producto de amplificación de ácido nucleico de una secuencia de ácido nucleico objetivo que es parte de un a plantilla de ácido nucleico. Por ejemplo, para determinar si la planta de soya que resulta de un cruce sexual contiene el evento transgénico MON89788 o si una muestra de soya colectada de un campo comprende MON89788, o un extracto de soya, tal como alimento, harina o aceite comprende MON89788. El ADN extraído de

una muestra de tejido vegetal de soya o extracto se puede someter a un método de amplificación de ácido nucleico usando un par cebador que incluye un cebador derivado de la región genómica adyacente al sitio de inserción de ADN transgénico heterólogo insertado, y un segundo cebador derivado del ADN transgénico heterólogo insertado para producir un amplicón que es diagnóstico para la presencia del ADN del evento. El amplicón es de una longitud y tiene una secuencia que también es diagnóstico para el evento. El amplicón puede variar en longitud de la longitud combinada de los pares cebadores más un par base nucleótido, o más aproximadamente cincuenta pares base nucleótido, o más aproximadamente doscientos cincuenta pares base nucleótidos, o más aproximadamente trescientos cincuenta pares base de nucleótidos o más.

Alternativamente, un par cebador se puede derivar de secuencia genómica de flanqueo en ambos lados del ADN insertado para producir un amplicón que incluye la secuencia nucleotídica de inserto completo. Un miembro de un par cebador derivado de la secuencia genómica vegetal se puede localizar a distancia de la molécula de ADN transgénica insertada, esta distancia puede variar de un par base nucleotídico hasta aproximadamente veinte mil pares base nucleotídicos. El uso del término "amplicón" excluye específicamente dímeros cebadores que se pueden formar en la reacción de amplificación térmica de ADN.

La amplificación de ácido nucleico se puede realizar por cualquiera de los varios métodos de reacción de amplificación de ácido

nucleico conocido en la técnica, incluyendo la reacción de cadena polimerasa (PCR). Una variedad de métodos de amplificación se conocen en la técnica y se describen, inter alia, en la Patente de E.U.A. Nos. 4,683,195 y 4,683,202 y en Innis et al. (1990). Los métodos de amplificación de PCR se han desarrollado para amplificar hasta 22kb de ADN genómico y hasta 42 kb de ADN bacteriófago (Cheng et al., 1994). Estos métodos así como otros métodos conocidos en la técnica de amplificación de ADN se pueden usar en la práctica de la presente invención. La secuencia del inserto de ADN heterólogo o secuencia de flanqueo del evento MON89788 de soya y se puede verificar, y corregir si es necesario por medio de amplificación de dichas moléculas del genoma del evento usando cebadores derivados de las secuencias provistas aquí seguido por métodos de secuenciación de ADN estándares aplicados al amplicón PCR o al ADN transgénico/genómico clonado aislado.

El amplicón producido por estos métodos se puede detectar por medio de una pluralidad de técnicas. Uno de dicho método es Genetic Bit Analysis (Nikiforov, et al., 1994) donde un oligonucleótido de ADN se diseña para traslapar tanto la secuencia de ADN genómico de flanqueo adyacente como la secuencia transgénica de ADN insertado. El oligonucleótido se inmoviliza en pozos de una placa de micropozo. Después de PCR de la región de interés (usando un cebador en la secuencia insertada y uno en la secuencia genómica de flanqueo adyacente), un producto PCR de cadena sencilla se puede hibridar al oligonucleótido inmovilizado y servir como una

plantilla para una reacción de extensión base sencilla usando un ADN polimerasa y ddNTP específicos etiquetados para la base siguiente esperada. La lectura puede ser fluorescente o con base en ELISA. Una señal indica la presencia de la secuencia genómica inserto/flanqueo debido a la amplificación exitosa, hibridación, y extensión de base sencilla.

Otro método es la técnica piro-sequenciación como se describe por Winge (2000). En este método un oligonucleótido se diseña para traslapar el ADN genómico adyacente y la unión de ADN inserto. El oligonucleótido se hibrida a un producto PCR de cadena sencilla de la región de interés (un cebador en la secuencia insertada y uno en la secuencia genómica de flanqueo) y se incuba en presencia de una ADN polimerasa, ATP, sulfurilasa, luciferasa, apirasa, adenosina 5' fosfosulfato y luciferina. Se añaden DNTP de manera individual y la incorporación resulta en una señal ligera que se mide. Una señal ligera indica la presencia de la secuencia inserto transgénico/flanqueo debido a la amplificación exitosa, hibridación, y extensión sencilla o multi-base.

La polarización de fluorescencia como se describe por Chen et al. (1999) es un método que se puede usar para detectar el amplicón de la presente invención. Usando este método se designa un oligonucleótido para traslapar el flanqueo genómico y la unión de ADN insertado. El oligonucleótido se hibrida a un producto PCR de cadena sencilla de la región de interés (un cebador en el ADN insertado y uno en la secuencia de ADN genómica de flanqueo) y se incuba en presencia de un ADN polimerasa y un ddNTP

etiquetado-fluorescente. La extensión de base sencilla resulta en la incorporación del ddNTP. La incorporación se puede medir como un cambio en la polarización usando un fluorómetro. Un cambio en la polarización indica la presencia del inserto transgénico/secuencia genómica de flanqueo debido a la amplificación, hibridación, y extensión de base sencilla exitosas.

Taqman® (PE Applied Biosystems, Foster City, CA) se describe como un método para detectar y cuantificar la presencia de una secuencia de ADN y se entiende completamente en las instrucciones proporcionadas por el fabricante. Brevemente, una sonda de oligonucleótido FRET se diseña para traslapar el flanqueo genómico y la unión de ADN inserto. La sonda FRET y los cebadores de PCR (un cebador en la secuencia de ADN inserto y uno en la secuencia genómica de flanqueo) se ciclan en presencia de una polimerasa termoestable y dNTP. La hibridación de la sonda FRET resulta en la escisión y liberación del radical fluorescente a distancia del radical de templado en la sonda FRET. Una señal fluorescente indica la presencia de la secuencia genómica de flanqueo/de inserto transgénico debido a amplificación e hibridación exitosas.

Guías moleculares se han descrito para usarse en detección de secuencia como se describe en Tyangi et al (1996). Brevemente, una sonda de oligonucleótido FRET se designa para traslapar la unión genómica de flanqueo y ADN inserto. La estructura única de la sonda FRET resulta en ésta conteniendo una estructura secundaria que mantienen los radicales fluorescentes y de templado en proximidad cercana. La sonda FRET y

cebadores PCR (un cebador en la secuencia de ADN inserto y uno en la secuencia genómica de flanqueo) se ciclan en presencia de una polimerasa termoestable y dNTP. Después de la amplificación de PCR exitosa, la hibridación de la sonda FRET a la secuencia objetivo resulta en la remoción de la estructura secundaria de la sonda y separación espacial de los radicales fluorescente y de templado. Una señal fluorescente resulta. Una señal fluorescente indica la presencia de la secuencia genómica de flanqueo/de inserto transgénica debido a amplificación e hibridación exitosas.

Otros métodos descritos, tales como, micro-fluidos (Publicación de Patente US 2006068398, Patente US No. 6,544,734) proveen métodos y dispositivos para separar y amplificar muestras de ADN. Colorantes ópticos usados para detectar y cuantificar moléculas de ADN específicas (WO/05017181). Dispositivos de nanotubo (WO/06024023) que comprenden un sensor electrónico para la detección de moléculas de ADN o nanoperlas que unen moléculas de ADN específicas y que pueden entonces ser detectadas son útiles para detectar moléculas de ADN de la presente invención.

Los equipos de detección de ADN se pueden desarrollar usando las composiciones descritas en la presente y los métodos descritos o conocidos en la técnica de detección de ADN. Los equipos son útiles para la identificación de ADN del evento de soya en una muestra y se pueden aplicar a métodos para la procreación de plantas de soya que contienen ADN. Los equipos pueden contener cebadores o sondas de ADN que son homólogos o

complementarios a SEQ ID NOs:1-4 y 9 o cebadores o sondas de ADN homólogos o complementarios a ADN contenido en los elementos genéticos transgénicos de ADN, estas secuencias de ADN se pueden usar en reacciones de amplificación de ADN o como sondas en un método de hibridación de ADN. La estructura del ADN de los elementos genéticos transgénicos contenidos en el genoma de soya y que se ilustra en la figura 1 comprende una región genómica 5' del genoma A3244 de soya que flanquea el inserto transgénico, el inserto comprendiendo una porción de la región de borde derecho (RB) de Agrobacterium tumefaciens, el promotor quimérico FMV/Tsf1 y elementos enlazados relacionados (Patente US 6,660,911; también referido como FMV/E1F1α) se conecta de manera operable a una secuencia de codificación de péptido tránsito de cloroplasto Arabidopsis EPSPS (en adelante referida como CTP2 o TS-AtEPSPS CTP2, patente US 5,633,435, conectada operablemente a un EPSPS resistente a glifosato (en adelante referido como CP4 EPSPS o aroA:CP4, aislado de la cepa CP4 Agrobacterium tumefaciens y secuencia de codificación modificada para expresión incrementada en células vegetales, Patente de E.U.A. 5,633,435), conectada operablemente a la región de terminación 3' de 1,5-bisfosfato carboxilasa ribulosa de arveja (en adelante referida como E9 3' o T-Ps.RbcS:E9, Coruzzi et al., (1984), una porción de la región (LB) de borde izquierdo de Agrobacterium tumefaciens, y la región genómica 3' del genoma A3244 de soya que flanquea el inserto transgénico. Moléculas de ADN útiles como cebadores en métodos de amplificación de ADN se pueden derivar de la

secuencia de los elementos genéticos del inserto transgénico contenido en el evento MON89788 de soya. Estas moléculas cebadoras se pueden usar como parte de un conjunto de cebadores que también incluye una molécula cebadora de ADN derivada del genoma de soya que flanquea el inserto transgénico. El evento MON89788 de soya se produce por medio de transformación de una línea de soya A3224 (Patente US 5,659,114) por medio de un método mediado por Agrobacterium, por ejemplo, métodos descritos en las Patentes US 6,384,301 y 7,002,058 (incorporada aquí para referencia en su totalidad).

Los inventores de la presente invención han descubierto que una línea de soya que comprende la región genómica tipo T MON89788 (tipo T es una combinación de un transgen y la región haplotipo asociada de un genoma vegetal) en su genoma tiene un rendimiento mejorado relativo a una línea que comprende la región genómica tipo T 40-3-2 previa. Esto se demuestra en los ensayos de campo replicados que incluyen datos de rendimiento colectados de locaciones múltiples en los Estados Unidos (solicitud de patente US 60/685584).

Los siguientes ejemplos se incluyen para demostrar ejemplos de ciertas modalidades de la invención. Se podrá apreciar por aquellos con experiencia en la técnica que las técnicas descritas en los ejemplos que siguen representan enfoques que los inventores han encontrado que funcionan bien en la práctica de la invención, y de este modo se pueden considerar que constituyen ejemplos de modos preferidos para su práctica.

Sin embargo, aquellos con experiencia en la técnica podrán, en vista de la presente descripción, apreciar que muchos cambios se pueden realizar en las modalidades específicas que se describen y obtener todavía un resultado similar o parecido sin desviarse de la esencia y alcance de la invención.

EJEMPLOS

EJEMPLO 1

Producción de amplicón diagnóstico para ADN genómico MON89788

ADN del evento MON89788 de soya transgénica se extrae de tejido que comprende semillas de soya, tejido vegetativo, o alimento. El ADN se aísla del tejido usando un Equipo Miniprep PLant DNeasy de Qiagen de acuerdo a las instrucciones del fabricante (Qiagen Corp. Valencia, CA).

Se produce un producto PCR que comprende una porción del ADN genómico que flanquea el extremo 5' de la inserción de ADN-T (ADN de transferencia que comprende el transgen) en el genoma de una planta que comprende MON89788. Este producto de ADN comprende SEQ ID NO: 3. PCR se puede realizar usando un cebador designado para hibridar a las secuencias de ADN genómico que flanquean el extremo 3' del inserto transgénico (cebador A de ADN, SEQ ID NO: 5; ver la figura 1) que forma par con un segundo cebador (cebador B de ADN, SEQ ID NO: 6) localizado en la región promotora transgénica (Patente US 6,660,911, SEQ ID NO: 28,

incorporada aquí para referencia y encontrada dentro de SEQ ID NO:9).

Se produce un producto PCR del extremo 3' del inserto transgénico que comprende una porción del ADN genómico que flanquea el extremo 3' de la inserción de ADN-T en el genoma de una planta que comprende MON89788. Este producto de ADN comprende SEQ ID NO: 4. PCR se puede realizar usando un cebador designado para hibridar a las secuencias de ADN genómico que flanquean el extremo 3' del inserto de cada evento (cebador D de ADN, SEQ ID NO: 8) y forma par con un segundo cebador (cebador de ADN C, SEQ ID NO: 7) localizado en la secuencia de terminación de transcripción T-Ps.RbcS:E9 3' en el extremo 3' del inserto.

La plantilla PCR incluye ~50 ng de ADN genómico. Como un control negativo ~50 ng de ADN genómico del cultivo de soya no transgénica se utiliza. Cada reacción PCR contiene 5 µl 10 X regulador de pH para REDAccutaq™ LA ADN Polimerasa Mix (Sigma-Aldrich, St. Louis, MO), 200 µM de cada dNTP (Sigma-Aldrich), 0.4 µM de cada cebador, y 2.5 Unidades de JumpStart™ REDTaq™ ADN Polimerasa (Sigma-Aldrich) en una reacción con volumen total de 50 µl. Las reacciones PCR se llevan a cabo bajo las siguientes condiciones de ciclación; 1 ciclo a 94°C durante 3 minutos (min); 32 o 35 ciclos a 94°C durante 30 segundos (s), 58°C durante 30 segundos, 72°C durante 30 segundos o 1 minuto; 1 ciclo a 72°C durante 10 minutos.

Se usan los pares de cebadores del evento de ADN para producir un amplicón diagnóstico para ADN genómico MON89788. Estos

pares cebadores del evento incluyen, pero no se limitan a cebadores A y B (SEQ ID NO: 5 y 6) y pares C y D de cebadores del evento (SEQ ID NO: 7 y 8), que se usan en el método de amplificación de ADN descrito. Además de estos pares cebadores, cualquier par cebador derivado de SEQ ID NO: 3 o SEQ ID NO: 4, o los complementos de estos, que cuando se usan en una reacción de amplificación de ADN produce un amplicón que comprende SEQ ID NO: 1 o SEQ ID NO: 2 diagnóstico para el tejido derivado del evento MON89788 de soya, respectivamente, se puede utilizar. Las condiciones de amplificación de ADN ilustradas en el cuadro 1 y cuadro 2 se pueden usar para producir un amplicón diagnóstico para MON89788 usando los pares de cebador de evento apropiados. Cualquier modificación de estos métodos usados para producir un amplicón diagnóstico para MON89788 se encuentra dentro de la experiencia ordinaria de la técnica. Un extracto que contiene putativamente ADN de una planta o semilla de soya que comprende MON89788, o un producto derivado de una planta que comprende MON89788 que cuando se analiza en un método de amplificación de ADN produce un amplicón diagnóstico para el evento de soya MON89788 se puede utilizar como una plantilla para la amplificación para determinar si está presente MON89788.

El amplicón es producido por el uso de al menos una secuencia cebadora derivada de SEQ ID NO: 3 o SEQ ID NO: 4 que cuando se usa en un método PCR produce un amplicón diagnóstico para el evento MON89788. Por ejemplo, la producción de los amplicones MON89788 se puede realizar

usando un Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, o un termociclador Eppendorf Mastercycler Gradient como se muestra en el cuadro 2, o por métodos y aparatos conocidos por aquellos con experiencia en la técnica.

CUADRO 1

Procedimiento PCR y condiciones de mezclado de reacción para la identificación de inserto transgénico/región de unión genómica 5' MON89788 de soya

Etapa	Reactivo	Cantidad	Comentarios
1	Agua libre de nucleasa	Agregar a volumen final de 20 µl	-
2	Regulador de pH de reacción 10X (con $MgCl_2$)	2.0 µl	Concentración final 1X de regulador de pH, 1.5 mM concentración final de $MgCl_2$
3	Solución 10 mM de dATP, dCTP, dGTP, y dTTP	0.4 µl	Concentración final 200 µM de cada dNTP
4	Cebador A del evento (SEQ ID NO: 5 re-suspendido en regulador de pH TE 1X o agua libre de nucleasa a una concentración de 10 µM)	0.2 µl	Concentración final 0.1 µM
5	Cebador A del evento (SEQ ID NO: 6 re-suspendido en regulador de pH TE 1X o agua libre de nucleasa a una concentración de 10 µM)	0.2 µl	Concentración final 0.1 µM

Etapa	reactivo	cantidad	Comentarios
6	RNasa, DNasa libre (500 μg/ml)	0.1 μl	50 ng/reacción
7	REDTaq ADN polimerasa (1 unidad/μl)	1.0 μl (recomendado para cambiar pipetas antes de la siguiente etapa)	1 unidad/reacción
8	ADN extraído (plantilla): - muestras a ser analizadas: *hojas individuales	 - 10-200 ng de ADN genómico	-
	*hojas agrupadas (máximo de 10 hojas/grupo)	-200 ng de ADN genómico	
	- control negativo	50 ng de ADN genómico de soya no transgénico	
	-control negativo	ADN no plantilla (solución en donde ADN se resuspende)	
	-control positivo	50 ng de ADN genómico de soya que comprende MON89788	

Si se necesita mezclado suave (sin calentar la parte superior en el termociclador), agregar 1-2 gotas de aceite mineral en la parte superior de cada reacción. Proceder con el PCR en un Strategene Robocycler (Stratagene, La Jolla, CA), MJ Engine (MJR-Biorad, Hercules, CA), Perkin

Elmer 9700 (Perkin-Elmer, Boeston, NA), o Termociclador Eppendorf Mastercycler Gradient (Eppendorf, Hamburgo, Alemania) usando los siguientes parámetros de ciclación (cuadro 2). El termociclador MJ Engine o Eppendorf Mastercycler Gradient se debe correr en el modo calculado. Se corre el termociclador Perkin-Elmer 9700 con una velocidad en rampa establecida en máximo.

CUADRO 2

Condiciones del termociclador

No de ciclo	Ajustes: Stratagene Robocycler
1	94°C 3 minutos
34	94°C 1 minuto 64°C 1 minuto 72°C 1 minuto y 30 segundos
1	72°C 10 minutos
No de ciclo	Ajustes: Engine MJ o Perkin-Elmer 9700
1	94°C 3 minutos
34	94°C 30 segundos 64°C 30 segundos 72°C 1 minuto
1	72°C 10 minutos
No de ciclo	Ajustes: Eppendorf Mastercycler Gradient
1	94°C 3 minutos
34	94°C 15 segundos 64°C 15 segundos 72°C 1 minuto
1	72°C 10 minutos

EJEMPLO 2

Determinación de secuencia de región transgénica/genómica y análisis Southern

La secuenciación de ADN de los productos PCR provistos para ADN que se puede usar para diseñar moléculas de ADN adicionales como cebadores y sondas para la identificación de plantas de soya o semillas que comprenden MON89788. Los productos PCR de los tamaños esperados que representan las secuencias transgénicas/genómicas 5' y 3' se aíslan por medio de separación de los productos PCR en un gel de agarosa 2.0% por medio de electrofóresis. Los productos PCR se aíslan incluyendo las regiones ADN 5' y 3' que extienden la unión de inserto entre la inserción transgénica en el genoma de soya. Los productos PCR 5´y 3' para MON89788 se purifican por medio de electrofóresis de gel de agarosa seguido por aislamiento de la matriz de agarosa usando el equipo de extracción de gel QIAquick (catálogo # 28704, Quiagen Inc., Valencia, CA). Los productos PCR purificados entonces se secuencian (por ejemplo, ABI Prism™ 377, PE Biosystems, Foster City, CA) y se analizan (por ejemplo, software de análisis de secuencia DNASTAR Inc., Madison, WI).

Se determina una secuencia de ADN para el segmento par base de nucleótido que representa la región transgénica/genómica del evento MON89788 como se ilustra en la figura 1 y se identifica como SEQ ID NO: 9. los elementos genómicos y transgénicos que están contenidos en SEQ ID NO:

9 se describen en el cuadro 3. Las regiones de flanqueo 5' y 3' están incluidas en SEQ ID NO: 9 y se proporcionan en SEQ ID NOs: 21 y 22.

Las secuencias de unión son moléculas de polinucleótido relativamente cortas que son secuencias de ADN novedosas y son diagnóstico para ADN de MON89788 cuando se detectan en un ensayo de detección de ácido polinucleico. Las secuencias de unión en SEQ ID NO: 1 y SEQ ID NO: 2 representan 10 polinucleótidos en cada lado de un sitio de inserción del fragmento transgénico y ADN genómico de soya en MON89788. Las secuencias de unión de polinucleótido más largas o más cortas se pueden seleccionar de SEQ ID NO: 3 o SEQ ID NO: 4. Las moléculas de unión (región de unión 5' SEQ ID NO: 1, y región de unión 3' SEQ ID NO: 2) son útiles como sondas de ADN o como moléculas cebadoras de ADN en métodos para la detección de ADN.

Los cebadores y sondas usados en un método Taqman® (Roche Molecular Systems, Inc., Pleasenton, CA) para la detección de una molécula de ADN de evento específica se desarrolla para el evento MON89788. Las moléculas cebadoras se refieren como SQ2824 (SEQ ID NO: 10), AQ2826 (SEQ ID NO: 11), SQ1141 (SEQ ID NO: 12), SQ1142 (SEQ ID NO: 13), SQ5543 (SEQ ID NO: 14) y las moléculas de sonda son referidas como PB871-6FAM (SEQ ID NO: 15), PB2191-VIC (SEQ ID NO: 16), y PB57-VIC (SEQ ID NO: 17). Los cebadores y sondas se usan en el método Taqman® de acuerdo a las instrucciones de los fabricantes para proveer un amplicón diagnóstico para ADN que comprende MON89788. Los tejidos de

soya que incluyen productos procesados, por ejemplo alimento, son fuentes útiles de ADN para este método. Los cebadores adicionales usados para producir un amplicón de alimento de soya incluyen SEQ ID NOs: 18-20.

CUADRO 3

Anotación del elemento genómico y genético del fragmento de ADN transgénico/ genómico (SEQ ID NO: 9) contenido en el genoma de soya que comprende MON89788

Elemento genético[1]	Locación en secuencia[2]	Función (referencia)
Secuencia de flanqueo del extremo 5' del inserto	1-1103	ADN GENOMICO DE SOYA
Región de unión 5'	1093-1113	Región de ADN que expande la inserción transgénica
Borde derecho-B[3]	1104-1145	Región de ADN de Agrobacterium tumefaciens que contiene la secuencia borde derecha usada para transferir del ADN-T (Depicker et al., 1982)
Secuencia de intervención	1146-1215	Secuencias usadas en clonación de ADN
P[4]-FMV/Tsf1	1216-2255	Promotor quimérico que consta de secuencias incrementadotas del promotor 35S del virus del mosaico escrofularia (Richins et al., 1987) y el promotor del gen Tsf1 de Arabidopsis thaliana (que codifica el factor de alargamiento EF-1 alfa (Axelos, et al., 1989)

Elemento genético[1]	Locación en secuencia[2]	Función (referencia)
L[5]-Tsf1	2256-2301	Líder no traducido 5' (exon 1) del gen Tsf1 de Arabidopsis thaliana que codifica el factor de alargamiento EF-1 alfa (Axelos et al., 1989)
L[6]-Tsf1	2302-2923	Intrón del gen Tsf1 de Arabidopsis thaliana que codifica el factor de alargamiento EF-1 alfa (Axelos et al., 1989)
Secuencia de intervención	2924-2932	SECUENCIAS USADAS EN CLONACION DE ADN
TS[7]-CTP2	2933-3160	Secuencias que codifican el péptido de tránsito de cloroplasto del gen ShkG de Arabidopsis thaliana que codifica EPSPS (Klee et al., 1987)
CS[8]-cp4 epsps	3161-4528	Secuencia de codificación optimizada de codón del gen aroA de la cepa Agrobacterium sp. CP4 que codifica la proteína EPSPS CP4 (Padgette et al., 1996; Barry et al., 1997)
Secuencia de intervención	4529-4570	Secuencias usadas en la clonación de ADN
T[9]-E9	4571-5213	Secuencia no traducida 3' del gen RbcS2 de Pisum sativum que codifica la sub-unidad pequeña Rubisco (Coruzzi et al., 1984)
secuencia de intervención	5214-5256	Secuencias usadas en la clonación de ADN
borde izquierdo -B	5257-5406	Región de ADN de Agrobacterium tumefaciens que contiene la secuencia borde izquierdo usada para transferencia del ADN-T (Backer et al., 1983)

Elemento genético[1]	Locación en secuencia[2]	Función (referencia)
Región de unión 3'	5396-5416	Región de ADN que expande la inserción transgénica
Secuencia de flanqueo del extremo 3' del inserto	5407-6466	ADN genómico de soya

Análisis Southern Blot

ADN genómico de una planta que comprende MON89788 y ADN genómico de soya de control (~15 μg de cada uno) se digiere con varias enzimas de restricción (140 U) en un volumen total de 150 μl incluyendo 15μl del regulador de pH del fabricante correspondiente (NEB, Beverly, MA). Las endonucleasas de restricción, por ejemplo, Bgl11, BamH1, Nco1, Hind111, y Bc11, se usan en el análisis Southern de MON89788. Las digestiones de endonucleasa se realizan a la temperatura apropiada por al menos 6 horas. Después de la incubación, el ADN se precipita con acetato de sodio 3M y 2.5 volúmenes de etanol. Posteriormente, el ADN se lava con etanol al 70%, se seca, y se resuspende en 40 μ1 de TBE. Regulador de pH de carga (0.2X) se añade a las muestras y después se somete a electrofóresis en geles de agarosa (0.8%) durante 16-18 horas a 30 volts. Los geles se tiñen con etidio-bromuro, después se tratan con una solución de despurinación (0.125 N HCl) durante 10 minutos, con una solución de desnaturalización (hidróxido de sodio 0.5M, cloruro de sodio 1.5M) durante 30 minutos, y finalmente con una

solución de neutralización (base Trizima 0.5 M, cloruro de sodio 1.5M) durante 30 minutos. El ADN se transfiere a la membrana Hybond-N (Amersham Pharmacia Biotech, Buckingamshire, Inglaterra) usando un Turboblotter (Schleicher y Schuell, Dassel, Alemania) durante 4-6 horas y después se fija a la membrana usando una luz UV.

Las membranas se pre-hibridan con 20 ml de solución DIG Easy Hyb (Roche Molecular Biochemicals, Indianápolis, IN; No. de cat. 1603558) durante 2-4 horas a 45°C. Las sondas de ADN radiactivas (^{32}P dCTP) homólogas o complementarias a SEQ ID NO: 1, o SEQ ID NO: 2, SEQ o ID NO: 3, o SEQ ID NO: 4, o una porción de las mismas se hace usando un equipo de etiquetado de ADN Radprime (Invitrogen, Carlsbad, CA; No. de cat. 18428-011). Los nucleótidos no incorporados se remueven usando columnas SEPHADEX G-50 (Invitrogen). La solución de pre-hibridación se reemplaza con 10 mls de solución DIG Easy Hyb pre-calentada que contiene la sonda desnaturalizada a una concentración final de 1 millón de conteos por ml. Las manchas se hibridan a 45°C durante 16-18 horas.

Las manchas se lavan con una solución poco severa (5X SSC, 0.1X SDS) a 45°C y después se lava repetidamente con una solución de severidad muy alta (0.1X SSC, 0.1% SDS) a 65°C. Las manchas se exponen a un tamiz de fósforo (Amersham Biosciences, Piscataway, NJ) por >2 horas y la lectura de exposición usando una máquina Data Storm 860 (Amersham Biosciences). Estos métodos y condiciones ejemplificados pueden ser modificados por aquellos con experiencia en la técnica de detección de ADN

en una muestra.

EJEMPLO 3

Control de maleza

El control del crecimiento de maleza en un campo de soya que comprende MON89788. Un campo se planta con semillas de soya que comprenden MON89788, las semillas se dejan germinar en plantas y el campo de las plantas se trata con una formulación herbicida que contiene glifosato. Una dosis efectiva de una formulación de glifosato a proporciones de tratamiento desde aproximadamente 0.1 kg ae/A (kg de equivalente ácido de glifosato/acre) a 1.36 o más kg ae/A se aplican al campo. Las proporciones aplicadas a veces varían desde aproximadamente 0.34 kg ae/A a 0.68 kg ae/A a una frecuencia de uno o más tratamientos durante la temporada de crecimiento como sea necesario para controlar el crecimiento de maleza en un campo. Las semillas de las plantas que comprenden MON89788 se recolectan de las plantas tratadas.

Un depósito del Monsanto Technology LLC, la semilla de soya representativa del evento MON89788 descrito anteriormente y recitado en las reivindicaciones se ha realizado bajo el Tratado de Budapest con la Colección de Cultivo Tipo Americana (ATCC), 10801 University Boulevard, Manassas, Va. 20110. El número de acceso ATCC para el depósito que comprende el evento MON89788 (también conocido como MON19788 o GM_A19788) es PTA-6708, depositado el 11 de mayo de 2005. El depósito se mantendrá en el

91

depositario durante un periodo de 30 años, o 5 años después de la última solicitud, o durante la vida efectiva de la patente, lo que sea más duradero, y se reemplazará según sea necesario durante ese periodo.

Habiendo ilustrado y descrito los principios de la presente invención, será aparente para aquellos con experiencia en la técnica que la invención se puede modificar en la disposición y detalle sin desviarse de dichos principios. Reclamamos todas las modificaciones que se encuentran dentro de la esencia y alcance de las reivindicaciones anexas.

Todas las publicaciones y documentos de patente publicadas citadas en esta especificación se incorporan aquí para referencia al mismo grado como si cada publicación o solicitud de patente individual fuera específicamente e individualmente indicada para ser incorporada para referencia.

Referencias

Las siguientes referencias, hasta el punto que provean procedimientos ejemplares u otros detalles complementarios a aquellos expuestos aquí, se incorporan específicamente en la presente para referencia.

U.S. Patent 4,535,060

U.S. Patent 4,683,195

U.S. Patent 4,683,202

U.S. Patent 5,094,945

U.S. Patent 5,463,175

92

U.S. Patent 5,554,798

U.S. Patent 5,633,435

U.S. Patent 5,633,435

U.S. Patent 5,659,114

U.S. Patent 6,040,497

U.S. Patent 6,384,301

U.S. Patent 6,544,734

U.S. Patent 6,660,911

U.S. Patent 6,660,911

U.S. Patent 6,689,880

U.S. Patent 6,689,880

U.S. Patent 6,733,974

U.S. Patent 6,740,488

U.S. Patent 6,740,488

U.S. Patent 6,818,807

U.S. Patent 6,825,400

U.S. Patent 6,893,826

U.S. Patent 6,900,014

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Solicitud U.S. 60/685584

Publicación U.S. 20040018518

Publicación U.S. 2006068398

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NOVEDAD DE LA INVENCION

REIVINDICACIONES

1.- Una secuencia de ácido nucleico que comprende la secuencia de SEQ ID NO: 1 o SEQ ID NO: 2.

2.- Una planta de soya o parte de la misma que comprende el evento MON89788, en donde la semilla de soya representativa que comprende el evento MON89788 se han depositado bajo el número de acceso ATCC PTA-6708.

3.- Una semilla de la planta de conformidad con la reivindicación 2, en donde la semilla comprende un evento MON89788.

4.- Un producto de consumo soya producido de la semilla de conformidad con la reivindicación 3.

5.- El producto de consumo de soya de conformidad con la reivindicación 4, caracterizado además porque se define como alimento, harina, hojuelas, o aceite.

6.- La parte de planta de soya de conformidad con la reivindicación 2, caracterizada además porque se define como una célula, polen, óvulo, flor, brote, raíz, u hoja.

7.- La planta de soya de conformidad con la reivindicación 2, caracterizada además porque se define como una planta de progenie de cualquier generación de una planta de soya que comprende dicho evento

96

MON89788.

8.- La planta de soya de conformidad con la reivindicación 2, caracterizada además porque el genoma de dicha planta comprende al menos una molécula de ADN seleccionada del grupo que consta de SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 21 y SEQ ID NO: 22.

9.- La planta de soya de conformidad con la reivindicación 2, caracterizada además porque el genoma de dicha planta produce un amplicón diagnóstico para el evento MON89788 cuando se analiza en un método de amplificación de ADN, dicho amplicón comprendiendo SEQ ID NO: 1 o SEQ ID NO: 2.

10.- La semilla de conformidad con la reivindicación 3, caracterizada además porque el ADN de la semilla produce un amplicón diagnóstico para el evento MON89788 cuando se analiza en un método de amplificación de ADN, dicho amplicón comprendiendo SEQ ID NO: 1 o SEQ ID NO: 2.

11.- El alimento, harina, hojuelas o aceite de conformidad con la reivindicación 5, caracterizado además porque se define comprendiendo un ácido nucleico que produce un amplicón diagnóstico para el evento MON89788 cuando se analiza en un método de amplificación de ADN, dicho amplicón comprendiendo SEQ ID NO: 1 o SEQ ID NO: 2.

12.- Una molécula cebadora polinucleotídica de ADN que comprende al menos 11 nucleótidos contiguos de SEQ ID NO: 3, o su complemento que es útil en un método de amplificación de ADN para producir

un amplicón diagnóstico para el evento MON89788.

13.- Una molécula cebadora polinucleotídica de ADN aislada que comprende al menos 11 nucleótidos contiguos de SEQ ID NO: 4, o su complemento que es útil en un método de amplificación de ADN para producir un amplicón diagnóstico para el evento MON89788.

14.- Un equipo de detección de ADN específico para el evento MON89788 que comprende al menos un ácido nucleico que comprende 11 o más nucleótidos contiguos homólogos o complementarios a SEQ ID NO: 3 o SEQ ID NO: 4.

15.- Un método para producir una planta de soya tolerante a herbicida de glifosato que comprende la introducción en el genoma de dicho evento MON89788 de planta.

16.- El método de conformidad con la reivindicación 15, caracterizado además porque se define comprendiendo las etapas de: (a) cruzar una primera planta de soya que comprende el evento MON89788 con una segunda planta de soya que carece del evento MON89788 para producir plantas de progenie; y (b) seleccionar al menos una primera planta de progenie que comprende dicho evento MON89788 y es tolerante a glifosato.

17.- El método de conformidad con la reivindicación 16, caracterizado además porque comprende auto-polinización de dicha primera planta de progenie para producir plantas de progenie de segunda generación y seleccionar al menos un primer homocigoso de planta para dicho evento MON89788.

99

18.- Un método para detectar la presencia de ADN correspondiente al evento MON89788 de soya en una muestra, el método comprendiendo: (a) poner en contacto una muestra que comprende ADN de soya con un conjunto de cebadores, que cuando se usan en una reacción de amplificación de ácido nucleico con ADN genómico del evento MON89788 de soya, produce un amplicón diagnóstico para el evento MON89788 de soya; y (b) realizar una reacción de amplificación de ácido nucleico, produciendo de este modo el amplicón diagnóstico; y (c) detectar el amplicón diagnóstico.

19.- Un método para detectar la presencia de un ácido nucleico correspondiente al evento MON89788 en una muestra, el método comprendiendo: (a) obtener una muestra de ADN de soya; y (b) analizar la muestra para la presencia de una secuencia de ADN del evento MON89788.

20.- El método de conformidad con la reivindicación 19, caracterizado además porque el análisis de la muestra de ADN comprende la detección de la presencia de la secuencia de ácido nucleico de al menos una de SEQ ID NO: 1 o SEQ ID NO: 2, o sus complementos.

21.- Una planta de soya que comprende una característica tolerante a glifosato que se enlaza genéticamente a una molécula de ácido nucleico que comprende SEQ ID NO: 1 o SEQ ID NO: 2.

22.- Un método para producir un producto de consumo de soya que comprende, (a) obtener la planta de soya o parte de la misma de conformidad con la reivindicación 2; y (b) producir un producto de consumo de soya de la planta de soya o parte de la misma.

40

23.- El método de conformidad con la reivindicación 22, caracterizado además porque el producto de consumo se define como alimento, harina, hojuelas, aislado de proteína, o aceite.

24.- Un método para el control del crecimiento de maleza en un campo que comprende plantas de soya que comprenden el evento MON89788, el método comprendiendo el tratamiento del campo con una cantidad de glifosato efectiva para controlar el crecimiento de la maleza, en donde las plantas de soya exhiben tolerancia al glifosato.

25.- El método de conformidad con la reivindicación 24, caracterizado además porque el tratamiento del campo se realiza desde la etapa V1 a R4 de crecimiento.

401

RESUMEN DE LA INVENCION

La presente invención se refiere a plantas de soya y semillas que comprenden el evento de transformación MON89788 y moléculas de ADN únicas para estos eventos; la invención también se refiere a métodos para detectar la presencia de estas moléculas de ADN en una muestra.

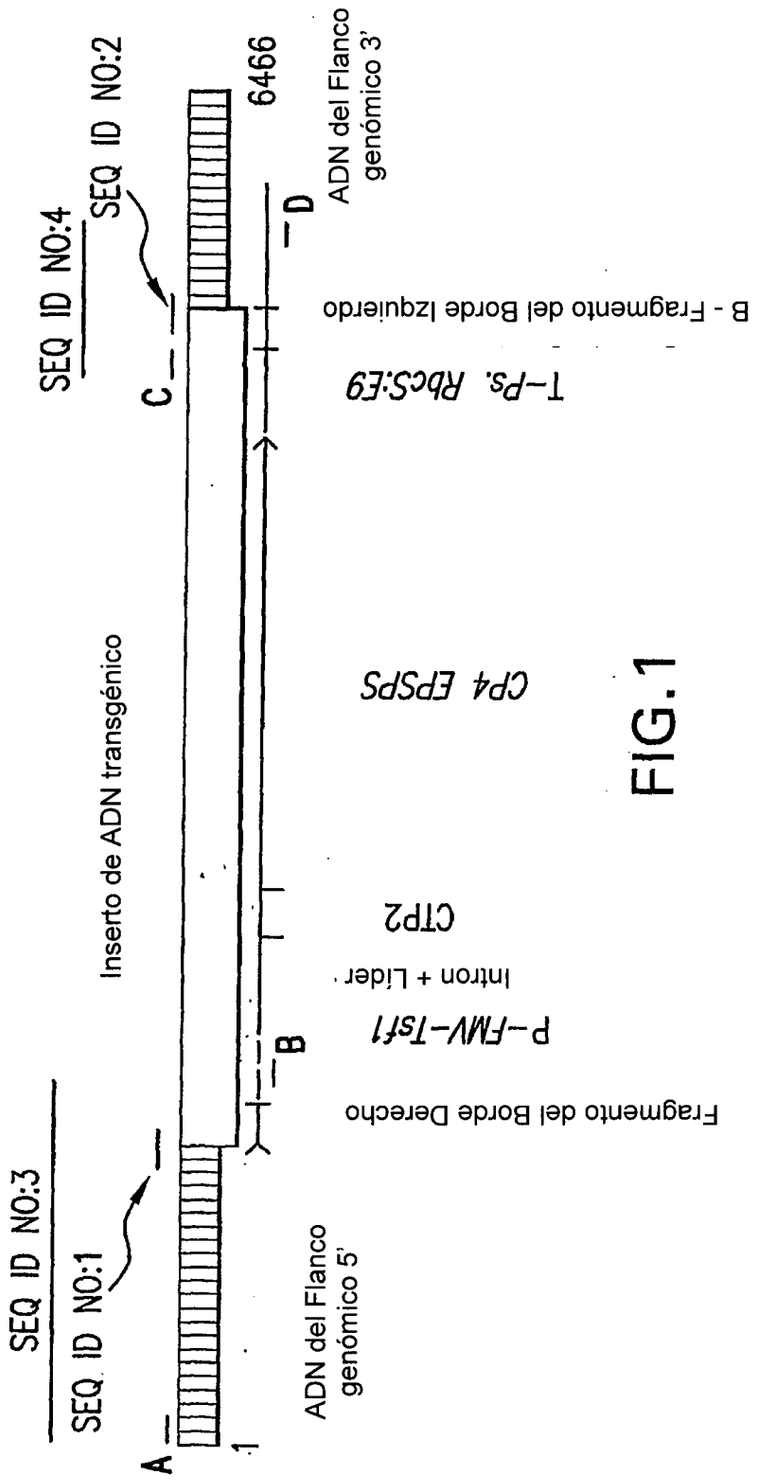

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SEQ ID NO:1
SEQ ID NO:4
SEQ ID NO:2
A
B
C
D
1
6466
ADN del Flanco genómico 5'
Fragmento del Borde Derecho
P-FMV-Tsf1
Intron + Líder
CTP2
CP4 EPSPS
T-Ps. RbcS:E9
B - Fragmento del Borde Izquierdo
ADN del Flanco genómico 3'
Inserto de ADN transgénico

FIG. 1

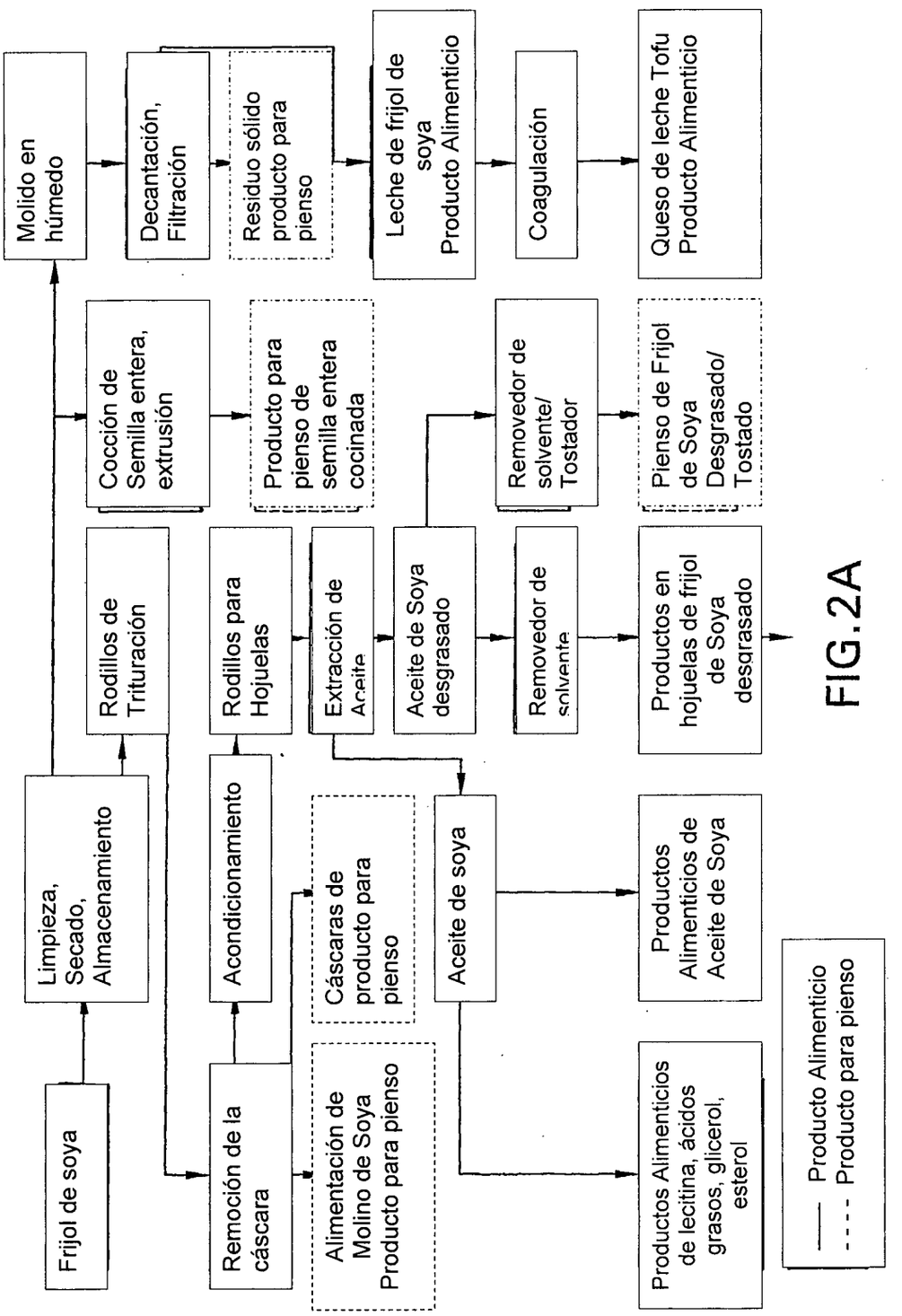

Frijol de soya
Limpieza, Secado, Almacenamiento
Molido en húmedo
Rodillos de Trituración
Cocción de Semilla entera, extrusión
Decantación, Filtración
Residuo sólido producto para pienso
Leche de frijol de soya Producto Alimenticio
Coagulación
Queso de leche Tofu Producto Alimenticio
Producto para pienso de semilla entera cocinada
Removedor de solvente/ Tostador
Pienso de Frijol de Soya Desgrasado/ Tostado
Remoción de la cáscara
Acondicionamiento
Rodillos para Hojuelas
Extracción de Aceite
Aceite de Soya desgrasado
Removedor de solvente
Productos en hojuelas de frijol de Soya desgrasado
Alimentación de Molino de Soya Producto para pienso
Cáscaras de producto para pienso
Aceite de soya
Productos Alimenticios de Aceite de Soya
Productos Alimenticios de lecitina, ácidos grasos, glicerol, esterol
Producto Alimenticio
Producto para pienso

FIG.2A

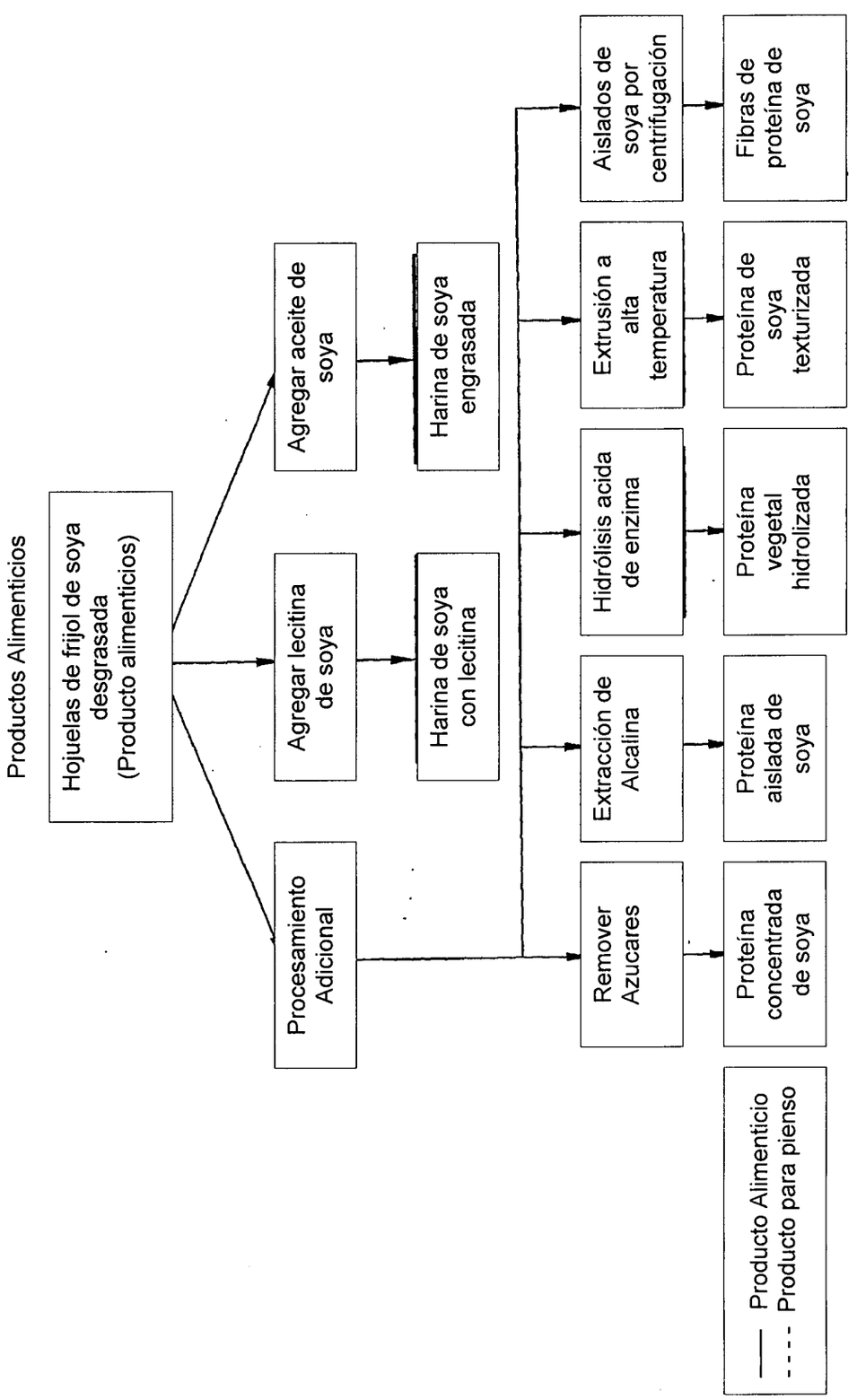

Productos Alimenticios
Hojuelas de frijol de soya desgrasada (Producto alimenticios)
Procesamiento Adicional
Agregar lecitina de soya
Agregar aceite de soya
Harina de soya con lecitina
Harina de soya engrasada
Remover Azucares
Extracción de Alcalina
Hidrólisis acida de enzima
Extrusión a alta temperatura
Aislados de soya por centrifugación
Proteína concentrada de soya
Proteína aislada de soya
Proteína vegetal hidrolizada
Proteína de soya texturizada
Fibras de proteína de soya
Producto Alimenticio
Producto para pienso

FIG.2B

105

LISTADO DE SECUENCIAS

<110> Malven, Marianne
Rinehart, Jennifer
Taylor, Nancy
Dickinson, Ellen

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<150> 2005-05-27

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AO-MONS:113US

IDS off Search Rpt. due 4-2-07

PATENT COOPERATION TREA

RECEIVED
F & J
AUSTIN INTL
JAN 11 2007

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
FULBRIGHT & JAWORSKI, LLP
Attn. Hanson, Robert E
600 Congress Avenue, Suite 2400
Austin TX 78701
ETATS-UNIS D'AMERIQUE

FULBRIGHT & JAWORSKI, LLP
IT DOCKETING
Docketed ☑ Not Req'd ☐ Confirmation ☐
Initials 1st RM Initials 2nd
JAN 05 2007
Attorney DLP/REH/JCC
Docket No AO-MONS:113WO
Action Req'd Date Due

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

Search + Opinion rec'd
Resp. to Opinion due 4-2-07 (Due 3 months from mail date)

(PCT Rule 44.1)

Applicant's or agent's file reference MONS:113WO	Date of mailing *(day/month/year)* 02/01/2007
	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US2006/020323	International filing date *(day/month/year)* 25/05/2006
Applicant MONSANTO TECHNOLOGY LLC	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

   Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Fascimile No.: (41–22) 338.82.70

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL–2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Andrea Pfau

Form PCT/ISA/220 (October 2005)

(See notes on accompanying sheet)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims,description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

PATENT COOPERATION TREA

PCT

126

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
MONS:113WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2006/020323	25/05/2006	27/05/2005
Applicant		
MONSANTO TECHNOLOGY LLC		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [X] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1
      [ ] as suggested by the applicant
      [X] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [ ] none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/020323

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [X] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in electronic form

   [X] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

nternational application No
PCT/US2006/020323

128

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01H5/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TERRY C F ET AL: "EVENT-SPECIFIC DETECTION AND ROUNDUP READY SOYA USING TWO DIFFERENT REAL TIME PCR DETECTION CHEMISTRIES" ZEITSCHRIFT FUER LEBENSMITTEL-UNTERSUCHUNG UND -FORSCHUNG. A, EUROPEAN FOOD RESEARCH AND TECHNOLOGY, SPRINGER, HEIDELBERG, DE, vol. 213, no. 6, November 2001 (2001-11), pages 425-431, XP001097295 ISSN: 1431-4630	1-25
Y	the whole document	1-25
Y	WO 02/44407 A2 (SES EUROP N V [BE]; WEYENS GUY [BE]; BARNES STEVE [BE]; ROSQUE INGE [B) 6 June 2002 (2002-06-06) page 2, paragraph 2	1-25
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 December 2006	02/01/2007
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Holtorf, Sönke

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2004/072235 A2 (MONSANTO TECHNOLOGY LLC [US]; CERNY R ERIC [US]; DUONG CAN [US]; HART) 26 August 2004 (2004-08-26) page 15 - page 16 the whole document	1-25
A	WINDELS P ET AL: "CHARACTERISATION OF THE ROUNDUP READY SOYBEAN INSERT" ZEITSCHRIFT FUER LEBENSMITTEL-UNTERSUCHUNG UND -FORSCHUNG. A, EUROPEAN FOOD RESEARCH AND TECHNOLOGY, SPRINGER, HEIDELBERG, DE, vol. 213, no. 2, August 2001 (2001-08), pages 107-112, XP001145520 ISSN: 1431-4630 the whole document	
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROTT M E ET AL: "Detection and quantification of roundup ready soy in foods by conventional and real-time polymerase chain reaction" JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, AMERICAN CHEMICAL SOCIETY. WASHINGTON, US, vol. 52, no. 16, 20 July 2004 (2004-07-20), pages 5223-5232, XP002405802 ISSN: 0021-8561 the whole document	
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INTERNATIONAL SEARCH REPORT

...information on patent family members

International application No
PCT/US2006/020323

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Eur Food Res Technol (2001) 213:425–431
DOI 10.1007/s002170100404

XP-001097295

ORIGINAL PAPER

P.D. M/2001
P. 425-431 (7)

Catherine F. Terry · Neil Harris

Event-specific detection of Roundup Ready Soya using two different real time PCR detection chemistries

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Abstract Recently, the DNA sequences of both the 5′ and 3′ sites of integration of the transgene in Roundup Ready soya have been described. We have utilised this information to design two event-specific assays for the quantitative detection of this genetically modified (GM) organism in food matrices using real time PCR. One assay has been designed to use TaqMan technology for detection using the ABI PRISM 7700 and the second to utilise Scorpion primers for use on the Roche Diagnostics LightCycler detection system. In both cases, the Roundup Ready gene was quantified relative to the endogenous soya lectin gene and results expressed as the percentage of GM soya relative to total soya content using standards of known GM content.

Keywords Quantitative real time PCR · Genetically modified organism detection · Roundup Ready soya · TaqMan · Scorpion

Introduction

Currently, within the EU there is a legislative requirement to label foods that contain, or are derived from, genetically modified organisms (GMOs) (Novel Food Regulation 258/97 and Regulations 1139/98/EG and 49/2000). For labelling, a threshold of 1% (w/w) of the ingredient has been set, above which GMO presence must be declared (Regulation 49/2000). The methodology to enable the detection and labelling of GMOs to accompany this legislation is based largely on the use of PCR to detect specific components of the inserted construct [1, 2, 3]. By amplification of transgene or construct-specific sequences it is possible to determine the presence or absence of approved GMOs in a wide range of food matrices.

C.F. Terry · N. Harris (✉)
BioAnalytical Innovation Team, LGC, Queens Road, Teddington, Middlesex, TW11 OLY, UK
e-mail: nh@lgc.co.uk
Tel.: +44-20-89437675, Fax: +44-20-89432767

Current PCR-based methods of detection amplify elements that may be present in the integrated constructs of several different GMOs. This, therefore, does not enable the discrimination of GMOs that contain the same inserted elements at different loci and the specific quantification of each in a sample that may contain a mixture of GMOs. In addition, it may not enable a test to distinguish between GMOs that are approved or unapproved within the boundaries of the EU. To enable unequivocal identification and quantification of a particular GMO, it is possible to target the PCR assay to amplify the DNA sequence at the integration border between the transgene construct and the plant genome, so called "transformation event"-specific PCR. Because integration of a transgene sequence into the plant genome is a random event it is highly unlikely to occur at the same genomic locus in two different GMOs, enabling the development of unambiguous detection assays specific for the transgenic construct and the integration site. Such an assay has recently been developed for the specific detection of Bt 11 maize [4].

The 5′ and 3′ junction regions at the sites of insertion of the transgene sequence in the genome of Roundup Ready soya have recently been characterised [5]. Our studies utilised the sequence data containing the 5′ junction (GenBank Accession AJ308514) to design and develop transgene event-specific assays for the quantitative detection of Roundup Ready soya. These were designed to be used for real time detection using quantification based upon measuring fluorescence during the log-linear phase of PCR amplification in which the resulting increase in fluorescence is proportional to the amount of specific PCR product. Two tests, one based upon the use of the TaqMan assay [6] and the other based upon a Scorpion assay [7] were developed for use on the ABI PRISM 7700 and -Roche Diagnostics LightCycler detection systems respectively.

The TaqMan assay is based upon the hybridisation of a dual (fluorophore and quencher) labelled probe to an amplicon [6]. Degradation of the hybridised probe by the exonuclease activity of Taq polymerase then separates

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amounts of primers and probes were also used in the same tube for the dual detection assay. dATP, dGTP and dCTP were each used at a concentration of 400 µM, 800 µM dUTP, 2.5 U of AmpliTaq Gold DNA polymerase, 0.5 U of AmpErase uracil *N*-glycosylase, 6 mM $MgCl_2$ and 1×TaqMan buffer A.

TaqMan probe reactions were run on the ABI Prism 7700 sequence detection system with the following thermal cycling protocol: 50 °C for 2 min, 95 °C for 10 min and 60 cycles of 95 °C for 15 s and 60 °C for 1 min.

Optimisation of the Scorpion assay. Amplification reactions (20 µl) were performed using the LightCycler Hybridisation Probes kit (Roche Diagnostics, UK) in conjunction with Taqstart antibody (Clontech, USA) according to manufacturer's instructions. Optimal amplification was carried out in glass capillaries in a final volume of 20 µl in 1×Master Mix containing 0.16 µl TaqStart antibody and either 2 mM $MgCl_2$ (lectin detection) or 6 mM $MgCl_2$ (Roundup Ready soya detection) and 250 nM primer and Scorpion probes in both cases. Samples were subjected to the following thermal cycling protocol: 95 °C for 120 s and 40 cycles of 95 °C for 0 s, 57 °C for 0 s, and 72 °C for 10 s at a ramp rate of 20 °C/s with data acquisition at 57 °C.

Determination of the detection limit of event-specific primers and probes. Sensitivity was tested on a serial dilution of DNA extracted from 5% (w/w) Roundup Ready soya flour using either the junction-specific Scorpion primer or TaqMan probe system. Samples were diluted to give a concentration range of 2025–0.25 copies of the Roundup Ready soya gene based upon 50 ng of soya DNA corresponding to approximately 50,000 copies of the haploid soya genome [12]. Each serial dilution was assayed in triplicate per PCR run and two separate runs were carried out in total for both systems.

Determination of the Roundup Ready soya content of food samples. With both detection systems triplicate amplification reactions were carried out on DNA from the certified reference materials to produce a calibration curve, plotting the logarithm of Roundup Ready soya concentration versus ΔCt (Ct_{GMO}–Ct_{Lectin}), where Ct-value can be defined as threshold cycle value. This is the PCR cycle at which a significant increase in PCR product is first detected. The equation of this standard curve was then used to determine the percentage of Roundup Ready soya relative to total soya for the two soya mixture test samples, using the ΔCt values obtained from triplicate analyses of these matrices.

Results and discussion

Reproducibility of the Scorpion and TaqMan assays

After optimisation of magnesium and primer/probe concentrations, each assay was applied to standards containing known percentages of Roundup Ready soya. Reproducibility of the threshold of detection (Ct) measurements was determined using 50 ng of total DNA varying in Roundup Ready soya content from 0–2% (w/w) per sample repeated in triplicate (Table 2, Table 3, Table 4).

Scorpion assays performed on the Roche Diagnostics LightCycler for lectin detection gave average Ct values varying from 22.61 to 24.37 cycles due to the fixed estimation of DNA content with a standard deviation of 0.07 to 0.43. For Roundup Ready soya detection, Ct values correlated with the amount of GM content present in each standard ranging from 29.97 to 35.86 cycles with a standard deviation of 0–0.9.

TaqMan assays for lectin detection gave average Ct values varying from 25.10 to 26.34 for the separate tube assay and 25.81 to 27.05 for the dual assay with standard deviations of 0.04–0.33 and 0.11–0.17 respectively. For Roundup Ready soya detection, Ct values correlated with GM content ranging from 31.52 to 35.90 cycles

Table 2 Reproducibility of the threshold of detection (*Ct*) measurements of replicate standards from 0–2% (w/w) Roundup Ready soya content using Scorpion detection on the Roche Diagnostics LightCycler. *GM* Genetically modified

GM content %	Ct value for reaction				
	1	2	3	Mean	SD
Total (Lectin)					
0.0	22.66	22.55	22.89	22.70	0.14
0.1	22.75	22.83	22.93	22.84	0.07
1.0	24.37	23.52	23.55	23.82	0.40
2.0	23.23	23.66	22.61	23.12	0.43
GM soya					
0.0	–	–	–	–	–
0.1	34.16	33.81	35.86	34.61	0.90
1.0	31.34	31.34	31.31	31.33	0.01
2.0	29.97	29.97	29.97	29.97	0

Table 3 Reproducibility of the Ct measurements of replicate standards from 0–2% (w/w) Roundup Ready soya content using separate TaqMan detection on the ABI Prism 7700

GM content %	Ct value for reaction				
	1	2	3	Mean	SD
Total (Lectin)					
0.0	24.93	25.19	25.17	25.10	0.11
0.1	25.40	25.43	25.47	25.43	0.33
1.0	26.08	26.82	26.13	26.34	0.34
2.0	25.38	25.34	25.29	25.34	0.04
GM soya					
0.0	–	–	–	–	–
0.1	35.30	35.72	36.69	35.90	0.58
1.0	33.12	33.06	33.16	33.11	0.04
2.0	31.53	31.24	31.79	31.52	0.22

Table 4 Reproducibility of the Ct measurements of replicate standards from 0 to 2% (w/w) Roundup Ready soya content using dual assay TaqMan detection on the ABI Prism 7700

GM content %	Ct value for reaction				
	1	2	3	Mean	SD
Total (Lectin)					
0.0	25.87	25.58	25.98	25.81	0.17
0.1	26.14	26.33	26.54	26.33	0.16
1.0	27.07	27.17	26.91	27.05	0.11
2.0	26.27	26.66	26.56	26.50	0.17
GM soya					
0.0	–	–	–	–	–
0.1	35.09	33.83	35.39	34.76	0.68
1.0	32.56	32.80	32.66	32.67	0.10
2.0	31.43	31.32	31.50	31.42	0.07

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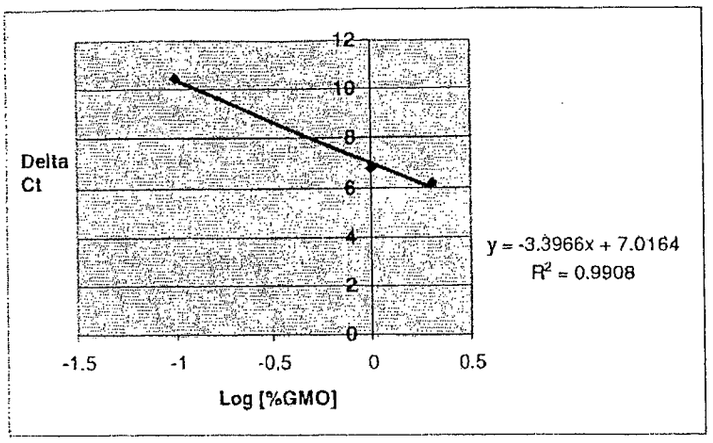


Fig. 2 Amplification plots for Roundup Ready soya detection in real time using the ABI Prism 7700 with a separate TaqMan assay, and standard curve, plotting log [GMO concentration] versus ΔCt

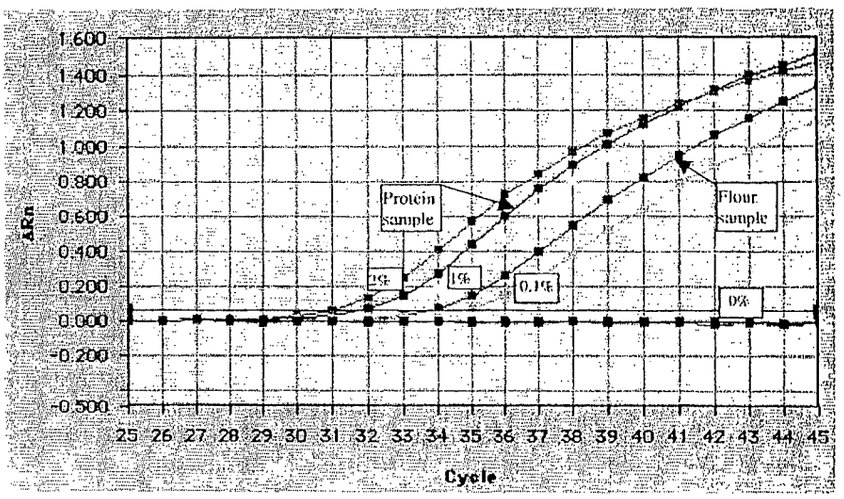


Table 6 Sensitivity of detection, in terms of copies of the Roundup Ready gene, using the TaqMan assay

Copy number	Ct value for reaction						Mean (of positives)	SD
	Experiment 1			Experiment 2				
2045	31.60	31.41	31.52	29.61	29.89	29.48	30.59	1.02
1023	32.58	32.54	32.48	30.67	30.94	30.87	31.68	0.94
205	35.11	35.21	35.11	32.82	33.41	33.01	34.11	1.14
20	39.02	37.87	39.36	36.52	36.18	37.33	37.71	1.18
2	–	42.13	–	–	40.24	–	41.12	0.94
0.2	–	–	–	–	40.85	38.91	39.88	0.97

Quantitative detection of Roundup Ready soya

The log of GMO concentration was plotted against ΔCt (Ct_{GMO}–Ct_{Lectin}) for each of the GMO standards to obtain a calibration plot for each assay. Using the Scorpion and TaqMan assays calibration lines with R^2 values of 0.99 were obtained for all three methods (Fig. 1, Fig. 2, Fig. 3).

Amplification reactions for two soya samples, one flour and one protein mix, assigned in a previous trial as containing 0.59% (w/w) and 1.30% (w/w) Roundup Ready soya respectively, were set up in triplicate for both systems and assayed for lectin and GM soya content. The ΔCt values for each sample were then applied to the standard calibration graphs obtained using Scorpion and TaqMan assays to obtain the calculated Roundup

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Testing of the detection limits of both assays using a dilution series down to 0.2 copies of the target gene showed that the TaqMan assay exhibits a slightly higher degree of sensitivity than that based on a Scorpion probe. In both cases, as would be expected, with decreasing target concentration there is a decrease in the number of amplifications yielding a positive Ct value. The absolute detection limit [9] for each assay would appear to be 2 copies of the target gene for the Scorpion assay and down to single molecule detection for that using TaqMan. However, to achieve this level of detection increasing numbers of amplifications would need to be carried out meaning that in practical terms the quantification limit [9] may be approximately 20 copies.

Methods for the real time quantification of GMOs rely upon the establishment of calibration curves using genomic reference material containing varying amounts of target material [10, 13]. The assays described generated calibration curves that exhibit a high degree of linearity making them suitable for the elucidation of GMO content in unknown samples. Using the calibration curves obtained it was possible to estimate Roundup Ready soya content in a soya flour and a soya protein mixture. The results obtained for the TaqMan assays showed a greater degree of accuracy for the calculated percentage GMO content of the soya flour sample with both separate and dual assays compared to the Scorpion assay. For the soya protein sample no great difference was seen with the three assays studied.

Two current studies have utilised either FRET or TaqMan assays on the Roche Diagnostics LightCycler for event-specific detection of Roundup Ready soya [8, 9]. The former method utilises the hybridisation of two probes (a donor and acceptor) to the amplicon and therefore needs four oligonucleotides (including amplification primers) to be designed and utilised in the PCR reaction. The use of Scorpions, however, represents an alternative method where only two oligonucleotides (the Scorpion probe and an unlabelled amplification primer) are needed in the reaction. This may therefore offer an alternative method for LightCycler-based detection where sequence-specific characteristics may put constraints upon FRET assay design.

Using the event-specific TaqMan probe in unison with the lectin probe previously described [11] it was possible to design a dual assay for simultaneous detection of Roundup Ready soya. With this system there appeared to be no loss in sensitivity, with dual detection giving a standard curve comparable to that obtained with the separate tube TaqMan detection and also a similar degree of response in terms of estimation of GM content. The use of dual detection offers the advantage of reducing the effects of any tube to tube variation that could occur if lectin and GM detection are carried out separately. In addition, assay costs are reduced as the amount of non-oligonucleotide reagents required to run an assay are reduced.

Use of the ABI 7700 platform has the advantage that the system uses 96 well plates, allowing replicate reactions for standards and samples for both lectin and Roundup Ready soya to be carried out at the same time, giving greater reproducibility. The Roche Diagnostics LightCycler, by comparison, only holds 32 samples with the result that runs requiring replication of standards and samples, such as is needed with GMO detection, have to be carried out in separate batches, potentially reducing result reproducibility. However, this has to be balanced against the fact that detection assays of the type described in this paper can be run on the LightCycler in less than 1 h compared to 3 h on the ABI PRISM 7700.

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Eur Food Res Technol (2001) 213:107–112
DOI 10.1007/s002170100336

ORIGINAL PAPER

Pieter Windels · Isabel Taverniers · Ann Depicker
Erik Van Bockstaele · Marc De Loose

XP-001145520

Characterisation of the Roundup Ready soybean insert

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Abstract In this article we describe the isolation and characterisation of the junction between insert DNA and plant DNA in the transgenic Roundup Ready soybean line event 40-3-2. Our results establish that during integration of the insert DNA several rearrangements occurred at the 3′ NOS junction and that the genomic plant DNA at the pre-integration site may have been rearranged. These findings highlight the utility of characterising junction regions to fulfil the request for information regarding which DNA sequences have been incorporated in commercialised transgenic lines. Furthermore, the characterisation of junction regions is, in our opinion, the method of choice to support method development for detection and identification of plant biotechnology-derived products.

Keywords Roundup Ready soybean · Junction fragment · Rearrangements · GMO · Identification

Introduction

Since labelling of foods containing genetically modified ingredients is mandatory in all European member states [1], the demand for reliable and easy to perform detection and identification methods for genetically modified organisms (GMOs) is high. As a governmental research institute involved in the development of strategies to detect and identify GMOs we analyse DNA sequences which flank the insert DNA in order to develop GMO event specific identification methods. Up to now most qualitative as well as quantitative GMO analysis methods make use of the polymerase chain reaction (PCR) to amplify and detect regulatory sequences that are frequently used in transgenic constructs, for example promoters, terminators and so on [2–6]. As a consequence, methods that amplify a unique sequence within the inserted DNA only allow detection of the presence of GMOs. Other methods are required for the identification of GMOs in food. It is clear that a primer pair that covers the junction between the insert DNA and the plant DNA will enable us to discriminate between different GMOs containing the same insert and to identify different GMOs containing different copy numbers of the same insert. As such, event specific primer pairs are the primers of choice in quantitative GMO analysis [7].

In Europe as well as in the United States a technical dossier containing all the information available and of importance to allow experts to judge the safety of a GMO has to be submitted in order to obtain authorisation to market a GMO[1, 8, 9]. Any new and relevant information that becomes available with regard to the technical dossier or to the risk assessment study has to be brought to the attention of the competent authorities. Here we show that the information obtained by characterising the junction region between plant DNA and insert DNA can be used to check the accuracy of the technical dossier or to complete already existing information. Padgette et al. [10] described earlier that the Roundup Ready (RR) single genetic insert, that was introduced into soybeans by the particle acceleration method, contains a portion of the cauliflower mosaic virus (CaMV) 35S promoter, the Petunia hybrida 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) chloroplast transit peptide (CTP), the CP4 EPSPS coding sequence and a portion of the 3′ non-translated region of the nopaline synthase gene terminator. However, up to now no information has

P. Windels · I. Taverniers · E. Van Bockstaele · M. De Loose (✉)
Department for Plant Genetics and Breeding,
Centre for Agricultural Research, Caritasstraat 21,
9090 Melle, Belgium
e-mail: m.deloose@clo.fgov.be
Tel.: +32-09-2722876, Fax: +32-09-2722901

A. Depicker
Department of Molecular Genetics, Ghent University,
K.L. Ledeganckstraat 35, 9000 Gent, Belgium

E. Van Bockstaele
Department for Plant Production, Ghent University,
Coupure Links 653, 9000 Gent, Belgium

I. Taverniers
Committee for Research and Services and Arts, Hogeschool Gent,
Voskenslaan 270, 9000 Gent, Belgium

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been available regarding the exact end-points of the insert DNA and the sequences following those end-points for RR soybean event 40-3-2.

We are interested in a more detailed characterisation of the insertion event, especially as the analysis of the transgene organisation in transgenic rice and oat that were transformed using particle bombardment showed that intact transgene copies are frequently accompanied by rearranged and truncated transgene fragments [11, 12]. Therefore, both regions adjacent to the end-points of the insert DNA of RR soybean event 40-3-2 and part of the pre-integration site were isolated using anchored PCR [13, 14] and adapter ligation PCR [15]. The anchored PCR approach is an AFLP related method that is based on a transposon display procedure [16]. Initially the DNA is digested using a restriction enzyme of choice. This restriction of the DNA is followed by a ligation step, during which an adapter is ligated to the restriction fragments. Subsequently a touchdown PCR reaction using an adapter primer and a radioactively labelled anchor primer – a primer that is homologous to the end region of the insert DNA – will amplify the junction fragment. In contrast to the transposon display method there is no selective pre-amplification step during anchored PCR. Once the junction fragment is isolated and re-amplified the sequence of the fragment can be determined. The adapter ligation PCR approach on the other hand amplifies the junction region using a nested PCR approach combining adapter primers and anchor primers after ligation of adapters to the generated restriction fragments.

Materials and methods

DNA isolation

Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen) starting with 20 mg of ground soybeans. Wild type DNA from the soybean cultivar A5403 was used as negative control DNA during all experiments.

Digestion and adapter ligation

Templates for the anchored PCR and adapter ligation PCR reactions were prepared as follows. Genomic DNA was digested using one of the six following restriction enzymes: MseI, MboI, BfaI, TaqI, EcoRI or HindIII. 250 ng of genomic DNA was digested with 2.5 U restriction enzyme in 25 µl reaction buffer (10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) for 2 h at 37°C, except for TaqI restriction that needed an incubation of 2 h at 65°C. In the latter case, the restriction mix was covered with mineral oil. Inactivation of the restriction enzyme by heating (70°C) the samples for 15 min was required only when MboI or EcoRI were used. After digestion, 25 µl of reaction buffer containing 50 pmol adapter in the case of MseI, MboI, BfaI or TaqI digestion and 5 pmol in the case of EcoRI or HindIII digestion, 1 U T4-ligase and 9.6 pmol ATP was added to the tube. This mixture was incubated for 2 h at 37°C. The structure of the adapters and the adapter primers that were used, was as described elsewhere [17, 18,15].

Anchored PCR reactions

All anchored PCR reactions were performed according to Theuns et al. [14]. The position of the anchor primers is presented in Fig. 1, a sequence overview is given in Table 1.

Adapter ligation PCR

The adapter ligation PCR that was used, was a modification of the protocol described by Spertini et al. [15]. Some adjustments were made in order to optimise the protocol for working with soybean. A first PCR amplification was performed as follows: 8 µl of ligation reaction, 1X PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 400 µM dNTP's, 0.25 µM primer AP1 and 0.25 µM of the anchor primer (Fig. 1; Table 1) were mixed in a total volume of 50 µl. The PCR profile for this first PCR amplification was as described [15] except that the second amplification step from this first PCR amplification was lengthened from 32 to 37 cycles. Spertini and co-workers suggested lengthening the second amplification step when working with DNA derived from species with large genomes. Secondary PCR amplifications were conducted with 1 µl of a 10-fold dilution of the primary PCR products. The PCR mixture that was used during secondary PCR amplifications was similar to that used during the first PCR amplification, except for the use of primer AP2 and a nested anchor primer (Fig. 1; Table 1). The second amplification step of the second PCR was lengthened from 22 cycles to 30 cycles. In some

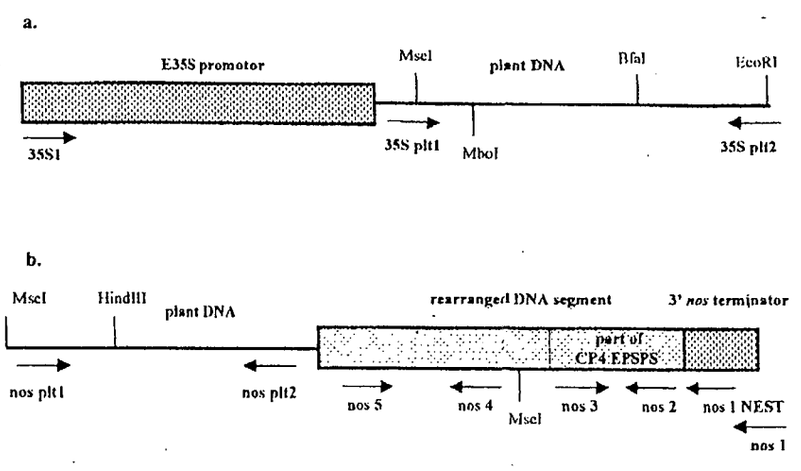


Fig. 1 Schematic representation of the amplified junction fragments (not drawn to scale). **a** structure of the amplified 35S promotor/plant DNA junction. **b** structure of the amplified 3'NOS/plant DNA junction. The position of the primers that were used to verify the structure of the junction fragments is depicted using arrows. Only those restriction sites that were important to amplify the junction fragments are presented.

Table 1 Sequence of the primers used in this study. For a schematic representation of the position of the different primers see Fig. 1

Primer name	Sequence (5'->3')
35S1 primer	GAT AGT GGG ATT GTG CGT CA
35S plt1 primer	GGT TCC CTA TGT TTA TTT TAA CCT G
35S plt2 primer	CCT TCA ATT TAA CCG ATG C
nos 1 primer	AGC GCG CAA ACT AGG ATA AA
nos 1 NEST primer	GCG CGG TGT CAT CTA TGT TA
nos 2 primer	CTT CAT GTT CGG CGG TCT
nos 3 primer	AAT CGT AGA CCC CGA CGA G
nos 4 primer	TGG GAA ATT TTA GCG AGA TTA T
nos 5 primer	TCT GGG AGA AGC AGT TAC TTA
nos plt2 primer	GAT CGG AGA AGA ACT GTT TGA
nos plt1 primer	GAG AAC TAC CTT CTC ACC GCA TT

cases two consecutive PCR cycles were not enough to generate an amount of amplified fragment that could be detected on agarose gel using ethidium bromide staining. In these cases a third PCR cycle was performed. Tertiary PCR amplifications were performed on 1 µl of the secondary PCR products. The same PCR mixture and primers were used as for the secondary PCR amplifications. The PCR profile was as follows: cycles 1–5: 25 s at 94°C and 3 min at 67°C followed by 35 additional cycles with the same denaturation temperature and an annealing/extension step of 60°C for 3 min. The PCR profile ended with a final extension step at 60°C for 7 min. 5 µl of the third PCR amplification product was analysed by means of 1.5% agarose gel electrophoresis and visualised with ethidium bromide.

Isolation of junctions

In order to isolate the correct junction from a dried polyacrylamide gel we superimposed the autoradiogram upon the gel. In this way the autoradiogram could be used as a template to find the fragment of interest on the gel. The desired junction was cut out of the gel using a sharp scalpel. The junction DNA was eluted from the gel slice by incubating the slice in 200 µl of water for 1 h on ice. During this incubation the solution was mixed 3-4 times. After a centrifugation of 10 min at maximum speed, 150 µl of the eluate was collected and could be used for further processing.

Junction fragments that were obtained by adapter ligation PCR were eluted from agarose gels by using the Qiaquick Gel Extraction Kit(Qiagen). All reactions were performed according to the manual.

Reamplification and sequencing of junctions

To reamplify each junction the same primers were used as those used to generate the junction fragment. The reamplification PCR was conducted using the following conditions: 1 µl of the eluted junction fragment, 1X PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 250 µM dNTP's, 0.4 µM anchor primer and 0.4 µM adapter primer. Water was added to a final volume of 50 µl. The PCR cycle profile used was the same as the one used during the anchored PCR. Cycle 1: 25 s at 93°C, 30 s at 65°C and 60 s at 72°C; cycle 2–13: 25 s at 93°C, 30 s at an annealing temperature 0.7°C lower than for each previous cycle, starting at 64.3°C, 55 s at 72°C; cycle 14–40: 25 s at 93°C, 30 s at 56°C and 55 s at 72°C. After completion of the PCR program, the concentration of the excised fragments was estimated on a 1.5% agarose gel, and the solution was concentrated to 25 ng/µl by means of ethanol precipitation. Subsequently the junctions were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The junctions were sequenced in both directions by using, on the one hand, the anchor primer and, on the other hand, the adapter primer as the sequencing primers. An ABI Prism 377 DNA Sequencer and matching software were used to separate the sequencing reactions and to analyse the sequence information.

BLAST analysis of amplified junction fragments

Homology analysis of the amplified junction fragments was done by performing a BLAST search against the GenBank sequence database [19].

Results and discussion

Characterisation of the 35S promotor/plant DNA junction (EMBL accession# AJ308514)

Initially we used the anchored PCR technique to amplify the junction between the 35S promoter region and the plant DNA. An anchor primer homologous to the 35S promotor region was designed. The position of the 35S1 primer is shown in Fig. 1. The anchor primer was used in six different anchored PCR reactions. Each reaction was performed on digested RR soybean DNA as well as on digested untransformed soybean DNA. The latter was used as a negative control. Using this strategy we succeeded in isolating and sequencing four different junction fragments spanning the junction between the 35S promoter and the plant DNA: a 35S/MseI fragment (234 bp), a 35S/MboI fragment (249 bp), a 35S/BfaI fragment (283 bp) and a 35S/EcoRI fragment (390 bp) (results not shown). A possible explanation for the absence of a 35S/TaqI and a 35S/HindIII fragment could be that the amplified junction fragments are too large to be detected on our polyacrylamide gel electrophoresis system. Subsequently, we analysed the sequence of the four amplified junction fragments. In order to verify the sequence data we reasoned that the sequences of the 35S/MboI, the 35S/BfaI and the 35S/EcoRI fragment should be an extension of the sequence of the 35S/MseI fragment. Indeed this was the case.

Since the 35S/EcoRI fragment was the longest fragment, it was most valuable for further analysis. Homology analysis made clear that the 35S/EcoRI fragment is made up of 181 bp of non-insert DNA and 209 bp of insert DNA (Fig. 2). As could be expected, the insert DNA is identical to the sequence of the CaMV 35S promotor. To verify if the 181 bp of non-insert DNA could be considered plant DNA we conducted a plant DNA-test-PCR reaction with a primer pair, the 35S plt1/35S plt2 primer pair (Table 1), that was located within this 181 bp sequence. The exact position of the primers that were used to establish whether plant DNA was present at the 35S/EcoRI junction is shown in Fig. 1. Since the expected PCR product was detected when using untransformed soybean DNA as template DNA we can conclude that the non insert DNA is homologous to a soybean plant DNA sequence.

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Fig. 2 Representation of the sequence of the amplified junction fragments. **a** sequence of the 35S junction fragment and **b** sequence of the 3′ NOS junction fragment. The insert DNA sequence is depicted in capitals, the plant DNA sequence in lower case, for the 3′NOS junction we used bold typing to indicate the part of the junction that is a part of the CP4 EPSPS coding sequence and the rearranged DNA segment is presented in slanted typing.

a.

```
   1 GATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCT 60
  61 TTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGTGCTCCTCGTGGGTGGGGGTCCATC 120
 121 TTTGGGACCACTGTCGGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATG 180
 181 GCATTTGTAGGAGCCACCTTCCTTTTCCAtttgggttccctatgtttattttaacctgta 240
 241 tgtatgatcttattttgaatgaaatgcaataagttatttctagtaaaaaaaaataaacat 300
 301 ttgatagaaacaaattaaagcatgcaaaaataactcattagcatcggttaaattgaaggg 360
 361 tttgaataatttgcacaaggttctgaattc 390
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b.

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   1 TGCGCGGTGTCATCTATGTTACTAGATCGGGGATCGATCCCCCACCGGTCCTTCATGTTC 60
  61 GGCGGTCTCGCGAGCGGTGAAACGCGCATCACCGGCCTTCTGGAAGGCGAGGACGTCATC 120
 121 AATACGGGCAAGGCCATGCAGGCCATGGGCGCCAGGATCCGTAAGGAAGGCGACACCTGG 180
 181 ATCATCGATGGCGTCGGCAATGGCGGCCTCCTGGCGCCTGAGGCGCCGCTCGATTTCGGC 240
 241 AATGCCGCCACGGGCTGCCGCTTGACCATGGGCCTCGTCGGGGTCTACGATTTCAAGCGC 300
 301 ATCATGCTGGGAAATTTTAGCGAGATTATAAGTATCTTCCTGGGGATCTCTGCTGTTACT 360
 361 GGTGAATAGTGAGACAGAGTCTTCTGAGCTCATAGGATAAAATAAATTATAATTAGTAAA 420
 421 TTTTTTAATTAAATAAATCAATTACTTCATAAATAATTTTTTTTATAGAATATGTTGACA 480
 481 TTCTAGCCGGATATAGAACTAATGTAAAGAAACCTTAAAAATTTTGTTTGGAAGAATATG 540
 541 TTATTGAAAGACAAATCTAATTAAGTTTATCAGGGTCATTTGTTGAAGATAGGAAACCTT 600
 601 CAGCAATTTGAATATTAAGTAACTGCTTCTCCCAGAATGATCGGAGTTTCTCCTCCTGCT 660
 661 ATTACATGAGCAAAAATAAAAAATAAATAAAAGATAAGATTAAGCTTCAACATGTGAAGG 720
 721 AGTAGTACACTCACCAGTGACCCTAATAGGCAACAGCATGAAAAAAAATAAAAAAGAATA 780
 781 AAAATAGCATCTACATATAGCTTCTCGTTGTTAGAAAAACAAAACTATttgggatcggag 840
 841 aagaactgtttgaggcgaatggcctggtcgtcgcggccatcgtcgagaagttcgtgaaga 900
 901 agctcgaatgcggtgagaaggtagttcttcttccaacagaaagttcaccacgcaattgcac 960
 961 agcgaagatctctccacgtccatttctctctctgtctctgatcttaagccattcattca 1020
1021 agacaagacaagagaagagaagaga 1045
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Characterisation of the 3′NOS/plant DNA junction (EMBL accession# AJ308515)

The junction between the 3′NOS region and the plant DNA was characterised using adaptor ligation PCR. Since no junction fragment connecting the 3′NOS region and the plant DNA could be amplified using one adaptor-aided PCR reaction, different sequential adaptor ligation PCR reactions had to be performed in order to reach the plant DNA.

The first adaptor-aided PCR reaction that we performed made use of nested anchor primers homologous to the 3′NOS region (Fig. 1; Table 1). The amplified fragment was sequenced and we tested if the DNA adjacent to the 3′NOS sequence was plant DNA. This experiment was done in the same way as described for the 35S junction fragment. A PCR reaction using a primer pair situated in the unknown DNA gave the expected fragment when using RR soybean DNA as a template, however when untransformed soybean DNA was used the expected amplification product could not be detected. This experiment shows that the DNA segment immediately adjacent to the 3′NOS region is not unaltered soybean plant DNA. For each fragment that was amplified during the sequential adaptor-aided PCR reactions, we tested if the amplified DNA showed homology to a soybean plant DNA sequence. All the primer pairs used to look for plant DNA homology are indicated in Fig. 1. The sequences of these primers is presented in Table 1. In the third sequential adaptor ligation PCR a fragment was amplified for which similarity to a soybean plant DNA sequence was proven using the plant DNA-test-PCR with the nos plt1/nos plt2 primer pair. After combining all sequence data for the different amplified fragments, we obtained a 1045 bp junction fragment at the 3′NOS region. This junction fragment is shown in Fig. 1. The next question that had to be answered was if the DNA segment that is present between the 3′NOS region and the plant DNA is homologous to a known sequence. A BLAST search gave no valuable homologies. When

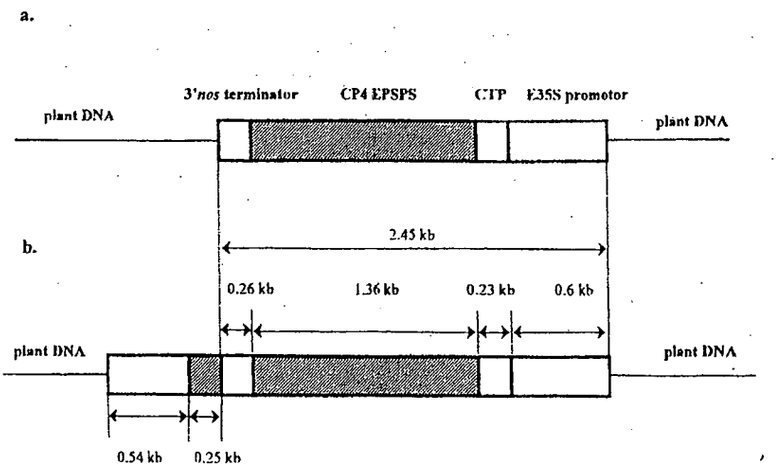


Fig. 3 A comparison between the insert structure as reported by a Padgette et al. [10] and b the structure of the insert deduced from the results described in this article

the sequence of the intermediate DNA segment was compared to the sequence of the insert DNA we found that a portion of 254 bp of the intermediate DNA segment was similar to a part of the CP4 EPSPS coding sequence (Fig. 2). This 254 bp DNA segment is situated immediately adjacent to the 3'NOS end point. Up to now no sequence homology could be found for the remaining 534 bp present between the plant DNA and the rearranged CP4 EPSPS segment.

To determine the exact starting point of the plant DNA at the 3'NOS junction we performed an anchored PCR reaction using a plant specific primer, the nos plt1 primer, as anchor primer. When using RR soybean DNA as a template this reaction should give a junction fragment, while a plant DNA fragment should be amplified when untransformed soybean DNA is used. Comparing both fragments should allow location of the exact starting point of the plant DNA. Indeed the two fragments were amplified and the exact starting point of the plant DNA could be fixed at position 829 of the 3'NOS junction fragment. In addition we analysed the sequence of the amplified plant DNA segment. First of all we compared the sequence of the amplified plant DNA fragment with the sequence of the plant DNA that is present adjacent to the 35S promotor end point. If no major rearrangements or deletions of the plant DNA have occurred during integration of the insert DNA, we can expect that both sequences should have some degree of similarity. However we could not detect any similarity between both sequences. To verify these results we performed a PCR reaction using a primer pair that consists of the 35S plt2 primer that is situated in the plant DNA adjacent to the 35S region and the nos plt1 primer that is situated in the plant DNA adjacent to the 3'NOS region. This primer pair should enable us to amplify the pre-integration site when using untransformed soybean DNA as template DNA, while the full insert should be amplified when using RR soybean DNA. Since no fragment was amplified when using untransformed soybean DNA, while for the RR soybean DNA the full insert DNA could be amplified, the above described findings are confirmed. An explanation for these findings could be that during integration of the insert DNA a large target site deletion occurred. Since a PCR setup suitable for amplification of DNA templates up to 12 kb was used, we expect a target site deletion of at least 12 kb. However, it is also possible that rearrangements of the plant DNA occurred due to the insertion of the insert DNA.

Taken together the results presented in this paper establish that at the 35S border no major rearrangements occurred during integration of the insert DNA and that plant DNA is present immediately adjacent to the 35S promotor end-point. This in contrast to the junction structure as detected at the 3'NOS junction. Adjacent to the 3'NOS region a 254 bp portion of truncated CP4 EPSPS coding sequence is present. This 254 bp DNA segment is followed by a DNA segment of 534 bp for which no sequence homology could be detected. Finally, adjacent to this unknown DNA segment we detected plant DNA. Furthermore, we can conclude that the target site was not simply disrupted by the insert but that during integration of the insert DNA rearrangements or a large deletion may have occurred.

In our opinion these results indicate that characterisation of junction fragments can offer important information for the accurate description of the insert structure of a transgenic line. Characterisation of junction fragments can be used to check the accuracy of technical dossiers on the one hand and on the other hand this method can be used to complete already existing information. A comparison between the results as reported by Padgette et al. [10] and our findings with regard to the structure of the RR insert is shown in Fig. 3. In the future, problems concerning the inaccurate description of transgene events can be avoided through a detailed characterisation of the transgene plant DNA junctions. Therefore, we welcome the idea that a detailed characterisation of the transgene plant DNA junction and of the plant target locus should be included in the technical dossier that is submitted to the competent authorities. These characterisations of the

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junction regions will also give the best information for the development of line specific identification methods, which are at least from a European standpoint of high importance in the context of food safety control.

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DEVELOPMENT OF A LINE SPECIFIC GMO DETECTION METHOD: A CASE STUDY

pp. 459-462 (4)

P. Windels*, I. Theuns*, J. Dendauw*, A. Depicker**, E. Van Bockstaele*,*** and M. De Loose*

* Department of Plant Genetics and Breeding, CLO, Caritasstraat 21, B-9090 Melle, Belgium
** Department of Molecular Genetics, University Gent, Flanders Interuniversity Institute for Biotechnology, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
*** Department of Plant Production, University Gent, Coupure Links 653, B-9000 Gent, Belgium

Summary

A strategy for a line specific GMO detection is presented. The method makes use of the anchored-PCR and can be used to screen foods for unauthorised GMOs. It is demonstrated that the proposed strategy is useful to develop a primer pair that can detect RoundUp Ready™ (Monsanto) soybean DNA in a line specific way.

Introduction

More and more foods are containing genetically modified organisms (GMOs) or GMO derived products. In the European Union such foods are regulated by regulation 90/220/EC and the directive Novel Food. These regulations stipulate a premarket approval and labelling requirements for foods derived from or containing GMOs. In this context, it is obvious that the authorities involved should have an adequate methodology for the identification of GMOs.

Up to now most DNA-based detection methods use the Polymerase Chain Reaction (PCR) to amplify parts of the inserted transgene. These PCR-based detection methods focus on frequently used genetic elements in GMOs, such as promoters, terminators, marker genes, etc. (Meyer, 1995; Pietsch *et al.*, 1997; Hupfer *et al.*, 1998). The disadvantage of such detection methods is that they are not line specific. This implies that it is impossible to discriminate between events that were obtained, using the same or similar transformation vectors.

Here we present a strategy to develop a line specific GMO detection procedure without preknowledge of the DNA sequence of the inserted fragment. The technique is based on the anchored-PCR (Theuns *et al.*, in preparation). This transgene display, used to amplify the junction between the insert and the plant-DNA, starts with digestion of the isolated DNA. By using a radioactively labelled anchor-primer, that is homologous with the border region of the inserted DNA, and an adapter-primer, that is specific for the adapter that is ligated at the end of each restriction fragment, the junction is specifically amplified. Subsequently the junctions are separated on a polyacrylamide gel after which the desired junction can be isolated from the gel. A method for the rapid isolation and subsequent sequencing of insert junctions is described. The obtained sequence data of the flanking plant-DNA of a particular T-DNA enabled us to design event-specific primers. Thereupon the newly designed event-specific primer together with the above mentioned anchor-primer is used in a regular PCR reaction to amplify an unique fragment spanning the junction between plant-DNA and the inserted DNA.

The technique is successfully being used to isolate and characterise the insert junction with plant-DNA of Monsanto's soybean line 40-3-2, a glyphosate tolerant soybean line, commercialised as RoundUp Ready (RR) soybeans. A line specific detection for RR soybeans is developed.

Materials and Methods

Anchored-PCR

The anchored-PCR was conducted as described by Theuns *et al.* (in preparation), using 5 different restriction enzymes – 1 hexacutter (EcoRI) and 4 tetracutters (MseI, MboI, BfaI and TaqI)

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– and the matching adapters and adapter-primers in combination with two anchor-primers: one homologous to the 35S promoter region (35S-primer) and one homologous to the nos 3' terminator region (3' nos-1-primer).

Isolation of junctions

In order to isolate the correct junction from the dried polyacrylamide gel, we superimposed the autoradiogram upon the gel. In this way the autoradiogram could be used as a template to find the fragment of interest on the gel. The desired junction was excised out of the gel using a sharp scalpel. The junction-DNA was eluted out of the gelslice by incubating the slice in 200 µl of water during 1 hour on ice. During this incubation the solution was mixed 3 to 4 times. After a centrifugation of 10 min. at maximum speed, 150 µl of the eluate was collected and could be used for further processing.

Reamplification and sequencing of junctions

Before reamplification the eluate had to be purified. For this purification the QIAquick PCR Purification Kit (Qiagen) was used.

The reamplification of each junction was done using the same primers as the primers that were used during anchored-PCR to generate the junction. The PCR was conducted using following conditions : 1µl of purified eluate, 1X PCR-buffer (Perkin Elmer, USA), 250 µM dNTP's (Pharmacia Biotech, USA), 0.4 µM anchor-primer and 0.4 µM adapter-primer. Water was added to a final volume of 50 µl. The PCR cycle profile used was the same as used during anchored-PCR. Cycle 1: 25s at 93°C, 30s at 65°C and 60s at 72°C; cycle 2-13: 25s at 93°C, 30s at an annealing temperature 0.7°C lower than for each previous cycle, starting at 64.3°C, 55s at 72°C; cycle 14-37: 25s at 93°C, 30s at 56°C and 55s at 72°C.

After completion of the PCR programme, the 50 µl reaction volume was loaded on a 1.5 % agarose gel. After electrophoresis the gel was stained with ethidium bromide and the reamplified fragments were visualised using an UV transilluminator. The reamplified fragments were excised from the agarose gel and the junctions were eluted from the gelslice using the QIAquick Gel Extraction Kit (Qiagen). The concentration of the excised fragments was determined on a 1.5 % agarose gel, and the solution was concentrated to 25 ng/µl by means of ethanol precipitation.

Subsequently the junctions were sequenced using the ABI Prism™ Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) according to the handbook provided with the kit. The junctions were sequenced in both directions by using on the one hand the anchor-primer and on the other hand the adapter-primer as the sequencing primers. An ABI Prism 377 DNA Sequencer and matching software were used to separate the sequencing reactions and to analyse the sequence information.

Recent results obtained with other junction fragments, show that it is possible to reamplify the junctions without performing a pre-purification step on the eluate.

Line specific RR soybean PCR

The line specific amplification of the DNA was carried out in a total volume of 25 µl using following conditions: 1X PCR-buffer (Perkin Elmer, USA), 200 µM dNTP's (Pharmacia Biotech, USA), 0.5 µM 35S-primer (5' GATAGTGGGATTGTGCGTCA 3'), 0.5 µM event-specific-primer (5' CCTTCAATTTAACCGATGC 3') and 25 ng of DNA. The PCR reactions were performed using the following cycle profile. Step 1 : initial denaturation at 95°C for 3 min. ; step 2 (34 cycles) : 20s at 95°C, 40s at 53°C and 40s at 72°C ; step 3 : 3 min. at 72°C. After the amplification 5 µl of the reaction mix was separated on a 1.5 % agarose gel and stained with ethidium bromide and visualised using a UV transilluminator.

Results and discussion

The proposed strategy was tested on a glyphosate tolerant soybean line that was constructed by Monsanto using the particle acceleration method. This RoundUp Ready soybean line 40-3-2 was

characterised at the molecular level (Padgette *et al.*, 1995). The results of this characterisation showed that line 40-3-2 contains part of the CaMV 35S promoter, the EPSPS chloroplast transit peptide from *Petunia hybrida*, the CP4 EPSPS gene and a portion of the 3' nontranslated region of the nopaline synthase gene terminator. Line 40-3-2 was selected because this line carried a single genetic insert (Fig. 1).

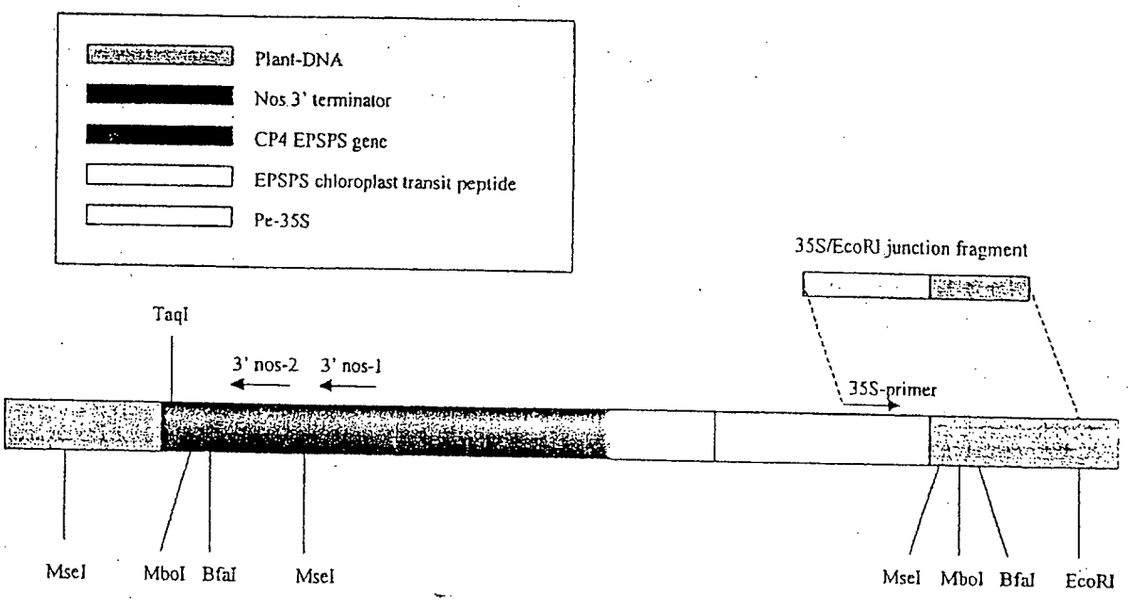


Fig. 1. Schematic diagram of inserted DNA present in RR soybeans (Padgette *et al.*, 1995).

To test our strategy we focussed on the 35S promoter region and the nos 3' terminator region because these two genetic elements flank the transgene and form the boundaries with the plant-DNA. In this way anchor-primers homologous to these two regions in combination with one of the 5 restriction enzymes and the matching adapter-primers would in all probability give us an anchored-PCR fragment that spans the border plant-DNA/inserted DNA. Because all tetracutters used had a recognition site between the 3'nos-1-primer and the insert border our work initially concentrated on the 35S junction fragment. Eventually the isolation of 4 different junction fragments spanning the junction 35S promoter/plant-DNA: 35S/MseI, 35S/MboI, 35S/BfaI and 35S/EcoRI, was successful. As could be expected the 35S/EcoRI fragment was the longest fragment. This 383 bp long 35S/EcoRI fragment was made up of 173 bp plant-DNA and 210 bp insert-DNA. We used this sequence information to design a primer that was situated in the plant-DNA. This newly designed event-specific primer and the 35S-primer make up a primer pair that should give a 360 bp amplicon when used to identify RR soybean DNA. Indeed the new primer pair was tested and gave a 360 bp fragment (Fig.2).

In the future this new primer pair will be tested on different foods containing soy (Messens *et al.*, in this volume). Also, the sequence of the 35S/EcoRI fragment will be used to develop a new line specific primer pair that will give a shorter amplicon (+/- 180 bp) to be able to identify RR soy DNA in processed foods in which the existing DNA could be fragmented (Hupfer *et al.*, 1998).

In the near future we will characterise and sequence also the 3'nos plant junction fragment. A new primer, the 3'nos-2 primer, homologous to the 3'nos terminator region, is being designed. As there is no MseI restriction site between the border and this new primer it should be possible to

amplify a 3'nos junction fragment. Once this junction fragment is isolated and sequenced, a primer specific for the plant-DNA adjacent to the nos 3' terminator will be developed. A combination of both event-specific-primers will be used in the future to amplify the pre-insertion locus in the wild type soybeans. A comparative analysis between the pre-insertion locus and the locus structure of the transgene locus could give us valuable information concerning the substantial equivalence between wild type and GMO.

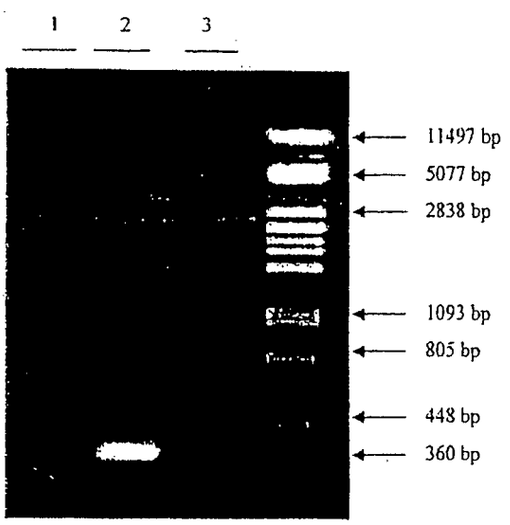


Fig. 2. Agarose gel analysis of line specific RR soy PCR products. Lane 1, line specific RR soy PCR on non-transgenic soy DNA. Lane 2, line specific RR soy PCR on transgenic RR soy DNA. Lane 3, negative control: line specific RR soy PCR on sample without DNA.

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Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection

Marta Hernández,[a] David Rodríguez-Lázaro,[b] Teresa Esteve,[a] Salomé Prat,[a] and Maria Pla[a,b,*]

[a] *Dpt. de Genètica Molecular, Institut de Biologia Molecular de Barcelona (IBMB), Centro de Investigación y Desarrollo-Consejo Superior de Investigaciones Científicas (CSIC), Jordi Girona 18-26, Barcelona 08034, Spain*
[b] *Institute of Food and Agricultural Technology (INTEA), University of Girona, Girona, Spain*

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Abstract

Commercialization of several genetically modified crops has been approved worldwide to date. Uniplex polymerase chain reaction (PCR)-based methods to identify these different insertion events have been developed, but their use in the analysis of all commercially available genetically modified organisms (GMOs) is becoming progressively insufficient. These methods require a large number of assays to detect all possible GMOs present in the sample and thereby the development of multiplex PCR systems using combined probes and primers targeted to sequences specific to various GMOs is needed for detection of this increasing number of GMOs. Here we report on the development of a multiplex real-time PCR suitable for multiple GMO identification, based on the intercalating dye SYBR Green I and the analysis of the melting curves of the amplified products. Using this method, different amplification products specific for Maximizer 176, Bt11, MON810, and GA21 maize and for GTS 40-3-2 soybean were obtained and identified by their specific T_m. We have combined amplification of these products in a number of multiplex reactions and show the suitability of the methods for identification of GMOs with a sensitivity of 0.1% in duplex reactions. The described methods offer an economic and simple alternative to real-time PCR systems based on sequence-specific probes (i.e., TaqMan chemistry). These methods can be used as selection tests and further optimized for uniplex GMO quantification.
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Keywords: GMO; Detection; Real-time PCR; SYBR Green I; Melting temperature

During recent years, a large number of genetically modified organisms (GMOs)[1] have been approved in the United States, the European Union, Canada, Japan, and other countries. The progressive tendency to increase both the cultivated areas and the number of modified traits is becoming evident [1]. The labeling normative [2,3] and the Japanese Ministries of Health and Welfare, and Agricultural Food and Fisheries [4] have encouraged the development and application of technologies aimed to detect and precisely identify the presence of GMO in food or food additives.

The most accepted GMO detection methods are based on DNA, because of the high stability of this molecule under the extreme conditions used during processing of some food products [5,6]. GMO detection through PCR relies on parallel amplification of the transgene and of an endogenous reference gene that provides a control both for the lack on inhibition and for the amplificability of the target DNA in the sample. Additionally, for quantitative analyses, amplification of the reference gene provides an estimation of the total amount of target DNA present in the sample. Several conventional PCR systems targeting different regions of the introduced genes in the maize "Maximizer" event 176 [7–9] and Bt11 [10] from Syngenta Seeds, the soybean GT 40-3-2 [11–14] or the YieldGard [15,16] and

* Corresponding author. Fax: +34-93-2045904.
E-mail address: mpsgmp@ibmb.csic.es (M. Pla).
[1] *Abbreviations used:* GMO, genetically modified organism.

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GA21 maizes from Monsanto [17], or the StarLink maize from Plant Genetics Systems [18] have been described. Moreover, conventional PCR systems suitable for use as species-specific endogenous controls have been published for soybean [19], maize [7], rapeseed [20], and potato and tomato [21,22].

The simultaneous amplification of multiple sequences in a single PCR is expected to save considerable time and effort in GMO detection analyses, as it should allow decreasing the number of reactions required to assess the presence of all possible GMOs [23]. Multiplex conventional PCR systems that are able to detect up to five different GMO targets in a single assay reaction have been reported [24–26]. The amplified fragments obtained in this assay are dissimilar enough to allow their identification in agarose gels, with fragments as long as 500 bp used in the amplification reaction. Though highly suitable for qualitative analyses, this method exhibits poor detection limits which could be improved by using more sensitive detection techniques such as real-time PCR. This technique [27] has the potential for sensitive and specific identification of target sequences, allowing their accurate quantification. Moreover, the lack of subsequent manipulation steps reduces the risks of cross-contamination, allowing high-throughput results with easy automation of the assay. Real-time PCR systems using the TaqMan chemistry to identify and quantify the maize Maximizer 176, Bt11, and MON810 events and the soybean GTS 40-3-2 event have been developed [16,17,28–34]. Nevertheless, implementation of these techniques to multiplexing real-time PCRs has not yet been done. Real-time PCR multiplex reactions would require the specific detection of the different fluorochrome dyes used to label each independent probe, which is difficult to achieve given the broad emission spectrum of the dyes used in real-time PCR assays [35,36]. Intercalating dyes such as SYBR Green I, with a high affinity for double-stranded DNA and enhanced fluorescence upon DNA binding [37], offer a good alternative as continuous monitoring of the amplified fragments' fluorescence along with gradient changes in temperature can be used to determine the melting curves of these fragments. Thereby, though fluorescence in the SYBR Green I reaction is not sequence specific, it is possible to identify the amplified products by their melting temperature (T_m) [38], thus making multiplexing feasible.

We report on the use of SYBR Green I-based melting curve determination assays in duplex PCRs targeted to (i) the GM-soybean GTS-40-3-2 and a soybean endogenous control and (ii) maize events Maximizer 176 and GA21. Furthermore, we used this method in a triplex reaction aimed to identify the maize events Maximizer 176, Bt11, and MON810. Using this technique, detection and identification of the PCR products could be attained without any need of further analyses, therefore these methods offer an efficient, reliable, and low-cost alternative to current methods for GMO identification.

Materials and methods

Plant materials

Powdered certified reference materials with Maximizer 176, Bt11, and MON810 maize lines and GTS 40-3-2 soy event were from the Institute for Reference Materials and Measurements and commercialized by Fluka (Buchs, Switzerland). Leaves from maize event GA21, *Lycopersicon esculentum* var. *Ailsa Craig*, *Solanum tuberosum* var. *Désirée*, *Arabidopsis thaliana* ecotype *Columbia*, *Brassica napus*, *Brassica oleracea*, *Helianthus annuus*, *Hordeum vulgare*, *Oryza sativa*, *Sorghum bicolor*, *Zea diploperennis*, and *Zea mays* cultivar W64A were from plants cultivated in green houses at the IBMB-CSIC. DNA extracted from *Secale cereale*, *Triticum aestivum*, *Panicum miliaceum*, *Glycine max*, *Lens esculenta*, *Vicia faba*, *Phaseolus vulgaris*, and *Lupinus albus* was provided within the framework of the "Qpcrgmofood" European project.

Plant DNA isolation and quantification

Genomic DNA was isolated from 0.1 g of plant material using a cetyltrimethylammonium bromide-based protocol as described [39]. DNA concentration was spectrophotometrically quantified with a GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). DNA concentrations were further assessed by agarose gel electrophoresis and ethidium bromide staining. UV-fluorescent emission was recorded and quantified using the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA) by comparison to known amounts of lambda phage molecular weight standards.

PCRs

Primers were purchased from MWG-Biotech AG (Ebensburg, Germany) (Table 1). PCRs were performed with the TaqMan PCR core reagents (Applied Biosystems–Roche Molecular Systems Inc., Branchburg, NJ) in 20 μL (uniplex and duplex reactions) or 50 μL (triplex reactions) of PCR mixture including 1× buffer II (100 mM Tris–HCl; pH 8.3, 500 mM KCl), 1× SYBR Green I (1:10,000) (Sigma, St. Louis, MI), 6 mM $MgCl_2$, 200 μM dNTPs, 1 U (uniplex and duplex reactions) or 1.5 U (triplex reactions) of Ampli*Taq* Gold DNA polymerase, 0.2 (uniplex and duplex reactions) or 0.3 (triplex reactions) units of AmpErase uracil *N*-glycosylase, and DNA template in the presence of adequate primers at the indicated concentrations. For uniplex reactions

Table 1
Oligonucleotides used in this study

Organism	Primers	Sequence (5′-3′)	Target gene	Size	Reference
Bt11 Maize	*Bt11f*	TCC TTG TCC TTG ACA CAG TTT CTG	*cryIA (b)*	75 bp	This work
	Bt11r	GAT GTC ACT AGT CCG AGA ACG AA			
Maximizer 176 maize	*crytm1*	GTG GAC AGC CTG GAC GAG AT	*cryIA (b)*	75 bp	[28]
	crytm2	TGC TGA AGC CAC TGC GGA AC			
GTS 40-3-2 Soybean	*sltmf3a*	GCA AAT CCT CTG GCC TTT CC	*CTP-EPSPS*	145 bp	[28]
	sltm2a	CTT GCC CGT ATT GAT GAC GTC			
GA21 Maize	*GA141F*	GGA TCC CCC AGC TTG CAT	*plasmid pDPG434 rice actin promoter*	72 bp	[17]
	GA212R	TTT GGA CTA TCC CGA CTC TCT TCT			
MON810 Maize	*35Saf2*	TGA TGT GAT ATC TCC ACT GAC G	*P-35S*	280 bp	[42]
	hsp508	CGG CAA GTA ATC AGC ACA G	*hsp70 intron*		This work
Maize	*ivr7*	GCG CTC TGT ACA AGC GTG C	*ivrA*	104 bp	[41]
	ivr8	GCA AAG TGT TGT GCT TGG ACC			
Soybean	*sltm1*	AAC CGG TAG CGT TGC CAG	*lectin*	81 bp	[28]
	sltm2	AGC CCA TCT GCA AGC CTT T			

amplimers were used at 300 nM in all cases; for soy duplex PCRs primers *sttmf3a* and *sltmr2a* were at 300 nM and *sltm1* and *sltm2* were at 50 nM; for duplex GA21 and Bt176 detection primers were used at 300 nM for *crytm1* and *crytm2* and at 50 nM for both *GA21* primers; for triplex PCRs amplimer *Bt11flr* was at 50 nM and *crytm1l2* was at 150 nM in combination with primer *35Saf2* at 200 nM and *monhsp508r* at 150 nM. SYBR Green I was diluted in autoclaved water to obtain a 10× concentration stock (1:1000). Reactions were run in an ABI PRISM 7700 Sequence Detection System device (Applied Biosystems), according to the PCR program: 2 min at 50 °C, 10 min at 95 °C, 50 (uniplex and duplex reactions) or 40 (triplex reactions) cycles of 15 s at 95 °C, and 1 min at 60 °C. After PCR amplification, T_m curve analysis was performed using the Dissociation Curve Analysis software v1.0 (Applied Biosystems). The PCR products were heated to 95 °C during 15 s, cooled at 60 °C for 20 s and then slowly heated back to 95 °C at a rate of 0.2 °C/s. Obtained fluorescence signals were continuously monitored during the slow warming-up gradient and showed a decreasing curve with a sharp fluorescence drop near the denaturation temperature. Plotting the negative derivate of the fluorescence over temperature versus the temperature ($-dF/dT$ versus T) generated peaks from which the T_m of the products were calculated. Unless specifically stated, all reactions were performed in triplicate.

Results and discussion

Design of the PCR systems

Seven different SYBR Green I-based real-time PCR systems were developed for GMO identification and optimized in uniplex format along a range of primer and SYBR Green I concentrations to exclude low-emission signals due to low dye concentrations or signal quenching due to a too-high concentration of the fluorochrome [40]. These PCR systems were targeted to the transgenic sequences of Roundup Ready soybean (Monsanto), maize Maximizer 176 and Bt11 (Syngenta Seeds), MON810 and GA21 (Monsanto), and the soybean *lectin* (AN:K0082) [28] and maize *invertase* (*ivrA*; AN:U16123) [41] reference genes. Primer pairs were those described in Table 1. Selection of primers was performed on the basis of the results previously obtained with conventional PCR and TaqMan real-time PCR systems, which evidenced the specificity of the transgene primers, and the suitability of the *ivrA* and *lectin* genes to be used as species-specific endogenous controls, as these genes do not show allelic variation among varieties and are present in the same number of copies in all cultivars. The specificity of each SYBR Green I assay system under the optimized PCR conditions (described under Materials and methods) was further assessed by running real-time PCRs on 50 ng of template DNA from six different GMOs and from 20 different plant species that were either evolutionarily related to maize or soybean or frequently found in food. No amplification with any of the plant DNA tested other than the corresponding maize or soybean GMO was observed, except for a faint signal (C_T above 35, corresponding to an underestimation of 10^5-fold) observed in the Bt11-specific reactions run on DNA template from Bt176 (data not shown). These results provide evidence that the optimized uniplex PCR systems are highly specific, as already reported.

In addition to primer specificity, the design of T_m multiplex assays requires that each different amplified DNA melts at a distinguishable temperature. Although

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current programs incorporate new parameters that allow quite accurate nearest-neighbor T_m estimation, the effect of certain PCR conditions is still empirical. Consequently, we ran quadruplicate real-time PCRs on 50 ng of template genomic DNA in the presence of SYBR Green I and experimentally tested the T_m of each amplified sequence (Fig. 1). We assigned each PCR product a confidence interval of experimental T_m calculated with a 95% confidence level. In addition, we experimentally assessed the T_m values of each amplified PCR fragment along a 5-log range of template DNA molecules. For all amplicons assayed, the experimental T_m obtained was in each case within the 95% confidence interval previously defined. Although it is widely accepted that increasing DNA concentration raises the T_m of dsDNA in solution [40], T_m analyses were performed at the end point of the optimized PCR and the T_m obtained did not deviate from the calculated confidence interval.

Multiplex PCR systems based on melting curve analysis

We assayed the suitability of the developed SYBR Green I real-time PCR systems to be used in a T_m melting curve determination multiplex format. After a careful analysis of the respective experimental T_m, we optimized two pairs of PCR systems in duplex format: (i) two GM-maize-directed PCR systems, i.e., Maximizer 176 and GA21; and (ii) a GM-soybean (GTS-40-3-2) and the soybean endogenous specific reference PCR systems. The T_m of these PCR systems differed by 5.1 and 5.6 °C, respectively, and with a 95% confidence interval did not overlap each other (Fig. 1). We optimized primer concentrations for each duplex PCR system in reactions containing equal amounts of the target DNAs. Optimal conditions (described under Materials and methods) were those producing similar end-point amounts of amplified DNA as calculated from the area of the melting curve derivative. As shown in Fig. 2, all target DNA molecules were correctly amplified in the duplex reactions and each specific amplicon was unambiguously distinguished by its T_m.

We also tested other possible pairs of PCR systems with smaller T_m differences in duplex format. We observed that, whereas amplicon pairs differing at least in 1.5 °C of T_m (Bt11- and GA21-specific amplicons) could still be identified in a duplex format, amplified DNA fragments with T_m means differing by only 1.2 °C (e.g., Bt11- and invertase-specific amplicons) produced single peak melting curves and thus were not suitable for use in duplex reactions (data not shown). This observation is in agreement with published data indicating that T_m differences as small as 2 °C are still detectable and give rise to distinguishable melting curves [38,40]. According to these results, a SYBR Green I-based melting curve determination multiplex assay should be usable for simultaneous identification of three PCR products, if the T_m of these products were compatible. To assay this possibility, we have used the Maximizer 176, the Bt11, and the MON810-specific PCR systems in a triplex format. The corresponding amplified fragments differ in their T_m, 3.6, 7.8, and 11.4 °C, respectively, and with 95% confidence intervals do not overlap with each other (Fig. 1). As shown in Fig. 2, using adequate concentrations of the primers, it was possible to unambiguously identify each specific amplicon by its respective T_m or melting curve, demonstrating that this technology can also be used in a triplex format.

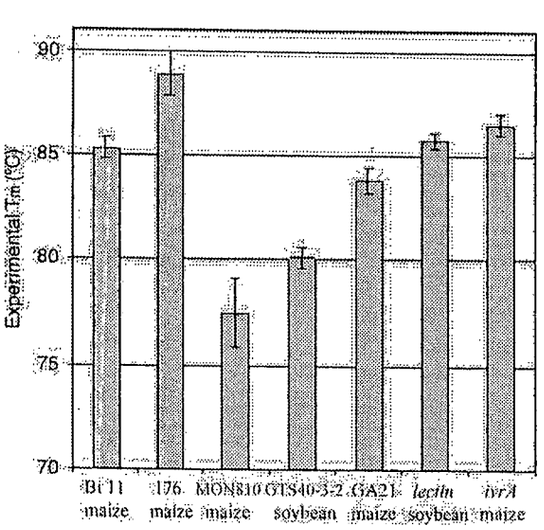


Fig. 1. Experimental T_m of the PCR product amplified from each organism and GMO. Confidence intervals are calculated with a 95% confidence level.

Sensitivity of the T_m multiplex PCR systems

After demonstrating the suitability of these methods not only in duplex but also in triplex format, it was important to assess the robustness of these multiplex PCR systems under conditions in which the starting amounts of the target DNA are quite different. With this aim, we tested the sensitivity of the two optimized duplex assay systems, i.e., those specific for Maximizer 176 and GA21 maize and for GTS 40-3-2 and non-GM soybean, under duplex PCRs that were set on different known ratios of the genomic DNAs purified from Maximizer 176 and GA21 maize and from GTS 40-3-2 soybean, respectively. We used reactions containing 50 ng of total template DNA in the following proportions: for Maximizer 176 and GA21 maize, 50, 10, 1, and 0.1% of each; for GTS 40-3-2 soybean, 5, 1, 0.5, and 0.1%. Whereas with the soybean duplex PCR system we were able to detect as slow as 0.1% GM-soybean in all replicates, for the Maximizer 176 and GA21 systems we detected 0.1% of any of the DNAs tested in only two of

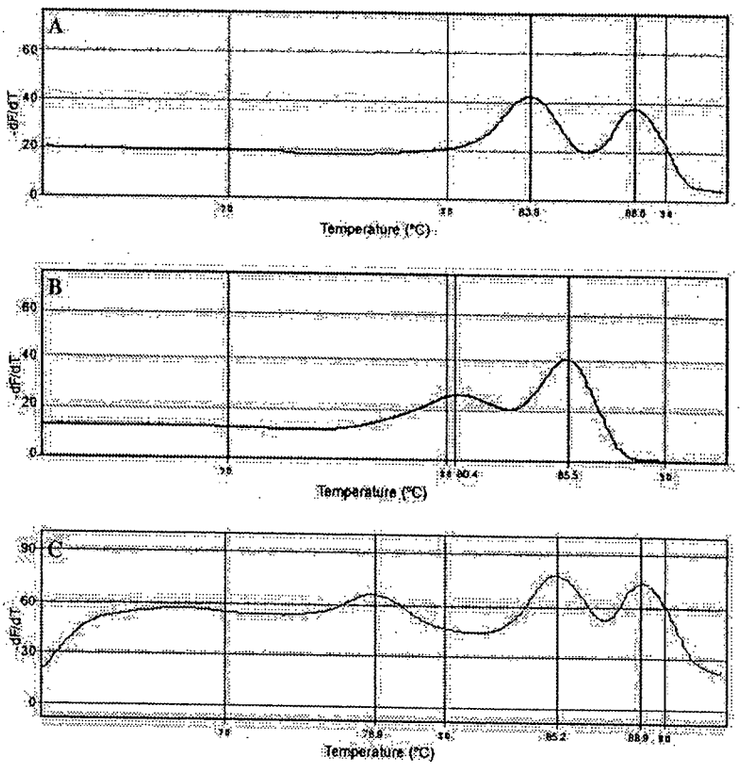


Fig. 2. Melting curve plot of the duplex GA21 and Maximizer 176 maizes (A), RR soya and lectin (B), and triplex Maximizer 176, Bt11, and MON810 maizes (C) PCR detection methods. In the x axis is represented the temperature in Celsius degrees and in the y axis the derivate of the fluorescence. The peaks indicate the melting points of the respective amplicons.

Table 2
Sensitivity of two T_m duplex PCR systems: Maximizer 176/GA21 transgenic maize lines and GTS 40-3-2 transgenic soybean line (*CTP-EPSPS* transgene and *lectin* gene)

Maximizer 176 maize (%)/GA21 maize (%)	0.1/99.9	1/99	10/90	50/50	90/10	99/1	99.9/0.1
Signal ratio Maximizer 176 maize	2/4	4/4	4/4	4/4	4/4	4/4	4/4
Signal ratio GA21 maize	4/4	4/4	4/4	4/4	4/4	4/4	2/4
GTS 40-3-2 soybean (%)	5	1	0.5	0.1			
Signal ratio *CTP-EPSPS* transgene	4/4	4/4	4/4	4/4			
Signal ratio *lectin* gene	4/4	4/4	4/4	4/4			

Signal ratio indicates positive signal for each of the target genes/total reactions (four replicates).

four replicates, with all reactions being positive when 1% target molecules were present in the sample (Table 2). Taken together, our results indicate that the developed real-time multiplex PCRs are able to detect Maximizer 176 and GA21 maize or GTS 40-3-2 soybean, with 1 and 0.1 sensitivity, respectively, which are limits about and lower than the threshold established by GMO regulations, e.g., Commission Regulation (EC) Nos. 49/2000 and 50/2000 [2,3]. Therefore, these newly developed melting curve-based real-time PCR systems would be adequate for use in routine GMO identification.

Use of T_m multiplex PCR systems in food samples

We have tested the applicability of the designed T_m multiplex real-time PCR systems for the analysis of mixed seeds or flour samples. Using the optimized conditions described in this report, it was possible to identify the *lectin* and the *CTP-EPSPS* genes present in the genomic DNA extracted from GTS 40-3-2 soybean seeds and flour; and the Maximizer 176, Bt11, and MON810 transgenes present in the DNA extracted from mixed samples of these maize GMO seeds or flour (data not shown). GMOs presence was further assessed by

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uniplex real-time PCR based on TaqMan chemistry [16,17,28], thus confirming the reliability of the melting curve-based real-time PCR assays.

Conclusions

To our knowledge, this is the first report on a T_m-based multiplex real-time PCR assay aimed at the detection of GMO in food. The T_m multiplex PCR systems described here are highly convenient for use in routine GMO identification analysis. Moreover, if GMO quantification is to be performed, they can be utilized as a detection system, to define the GMOs present in the sample and therefore the appropriate quantification methods to be applied. The sensitivity limits of the reported methods were 1% for Maximizer 176 and GA21 maize, and 0.1% for GTS 40-3-2 soybean, which are about and lower than the threshold established by GMO regulations. Therefore, when further optimized these SYBR Green I-based real-time PCR methods are suitable to be used for specific DNA quantification.

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Detection and Quantification of Roundup Ready Soy in Foods by Conventional and Real-Time Polymerase Chain Reaction

MICHAEL E. ROTT,* TRACY S. LAWRENCE, ERIKA M. WALL, AND MARGARET J. GREEN

Centre for Plant Health, Canadian Food Inspection Agency, 8801 East Saanich Road, Sidney, British Columbia, Canada V8L 1H3

Transgenic soybean line GTS-40-3-2, marketed under the trade name Roundup Ready (RR) soy, was developed by Monsanto (USA) to allow for the use of glyphosate, the active ingredient of the herbicide Roundup, as a weed control agent. RR soy was first approved in Canada for environmental release and for feed products in 1995 and later for food products in 1996 and is widely grown in Canada. Consumer concern issues have resulted in proposed labeling regulations in Canada for foods derived from genetically engineered crops. One requirement for labeling is the ability to detect and accurately quantify the amount of transgenic material present in foods. Two assays were evaluated. A conventional qualitative Polymerase Chain Reaction (PCR) assay to detect the presence of soy and RR soy and a real-time PCR to quantify the amount of RR soy present in samples that tested positive in the first assay. PCR controls consisted of certified RR soy reference material, single transgenic soybeans, and a processed food sample containing a known amount of RR soy. To test real-world applicability, a number of common grocery store food items that contain soy-based products were tested. For some samples, significant differences in amplification efficiencies during the quantitative PCR assays were observed compared to the controls, resulting in potentially large errors in quantification. A correction factor was used to try to compensate for these differences.

KEYWORDS: Soybean; Roundup Ready; quantification; real-time PCR; processed foods: genetically modified

INTRODUCTION

The European Union (EU) has had regulations for the labeling of novel foods since 1997. As a consequence, research on the most efficient, reliable, and accurate methods of detecting and quantifying genetically modified (GM) ingredients has been intense (*1*−*12*). Current EU regulations stipulate that products containing an ingredient of which ≥0.9% originates from a GM product must be labeled. For example, if a pure soy product contains ≥1% soy derived from a GM soy variety, it must be labeled, but if it contains only 0.5%, it would not have to be labeled. If the product consisted of only 10% soy and 90% other, but 1% of the soy present was derived from a genetically GM soy variety, it would also require labeling, even though on a weight per weight basis it contains less GM soy than the pure soy product containing 0.5% GM soy that does not require labeling. No mandatory labeling legislation has been implemented in Canada to date. However, the Canadian Food and Drug Act allows for the voluntary labeling of biotechnology-derived foods. Currently, the Canadian General Standards Board (CGSB) in conjunction with the Canadian Council of Grocery Distributors has developed a National Standard for Canada for the voluntary labeling of foods and food ingredients obtained from biotechnology (*13*). The aim of the CGSB is to develop standards, based on recognized international protocols, that will provide a model for label declarations that are understandable and not misleading for Canadian consumers. Their goal is also to establish procedures to distinguish biotechnology-derived foods from conventionally produced foods from production to retail and to establish the use of testing and monitoring procedures. The CGSB does not legislate labeling requirements.

In Canada, there are over 40 plant products containing unique transgenic DNA events approved for food (*14*). Of these, one of the most widely grown in Canada, in 2002, is herbicide-tolerant (GTS-40-3-2) soybean (*15*), with over 100 varieties containing this event registered in Canada. GTS-40-3-2, also known as Roundup Ready (RR), was developed by Monsanto and confers tolerance to the glyphosate-based herbicide Roundup. The GTS-40-3-2 event consists of an enhanced cauliflower mosaic virus 35S promoter, a CTP4 leader sequence from *Petunia hybrida*, and the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene conferring the herbicide tolerance followed by an *Agrobacterium tumefaciens* nopaline synthase terminator (*15*, *16*). A number of methods have been developed for the detection of RR soy. These include protein-based methods for the detection of the EPSPS gene product in

* Corresponding author [telephone (250) 363-6650, ext. 263; fax (250) 363-6661; e-mail rottm@inspection.gc.ca].

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Table 1. Soy Foods Used in This Survey

food	degree of processing	form of soy ingredient	food	degree of processing	form of soy ingredient
miso	high	fermented beans	boiled soybean	low	whole bean
soy sauce	high	fermented beans	dried (organic) soybean	low	whole bean
natto	high	fermented beans	roasted soy nuts	low	whole bean
TVP[b]	high	tsp[c]	soy nut snack	low	whole bean
meat alternative 1	high	tsp/soy sauce	soy nut spread	low	whole bean
meat alternative 2	high	tsp	simulated bacon bits	low	flour/soy protein/hsp
cheesies	high	hsp[d]	protein bar 2	low	flour/lecithin/isp
cracker 1	high	hsp	biscuit	low	flour
gravy mix 1	high	oil/hsp	gravy mix 2	low	flour/hsp
frozen dessert	moderate	soy powder/isp	bread 1	low	flour/isp/lecithin
soup	moderate	soy milk	bread 2	low	defatted flour
soybean pâté	moderate	protein	soup mix	low	soy powder
protein bar 1	moderate	roasted soybean/isp[a]	nutritional bar	low	flour/isp/soybean/lecithin/soy butter
soy beverage	moderate	soy milk	cheese	low	flour/oil
meal replacement beverage	moderate	protein	crispbread	low	defatted flour
coffee whitener	moderate	whole bean	cracker 2	low	flour/lecithin
yogurt	moderate	soy milk	flour	low	flour
chocolate pudding	moderate	soy milk			
meat alternative 3	moderate	spc[e]/isp			
tofu	moderate	soybean curd			
infant formula 1	moderate	oil/isp/soy milk			
infant formula 2	moderate	oil/isp/soy milk			

[a] isp, isolated soy protein. [b] TVP, textured vegetable protein. [c] tsp, textured soy protein. [d] hsp, hydrolyzed soy protein. [e] spc, soy protein concentrate.

transgenic raw or unprocessed soy products (*15*, *17*−*19*) and PCR-based methods, both qualitative and quantitative, which can also be used for the more highly processed soy-based food products (*2*, *5*, *6*, *8*−*10*, *15*, *17*, *20*−*22*). Several of these methods have been officially approved or validated by other government agencies (*2*, *15*, *18*, *23*). In this study a variety of foods containing soy ingredients that are available in the local supermarkets were sampled using two different PCR protocols. Our goal was to first establish that soy DNA and Roundup Ready (RR) DNA could be detected in the foods by qualitative PCR, targeting the endogenous soy lectin gene and the CTP4−CTP EPSPS junction of the RR gene (*15*, *17*, *24*), and to determine from what type of foods it was possible to amplify soy DNA and to get an initial impression on the prevalence of genetically modified soy in common food products. Those products testing positive were then further analyzed by real-time quantitative PCR, targeting different regions but the same lectin and RR genes (*15*, *25*) to determine the level of Roundup Ready soy in these foods. Few studies to date have attempted to quantify the amount of transgenic material in processed foods to determine the practical feasibility of such testing.

MATERIALS AND METHODS

Soy Food Samples. A selection of 39 soy food products including vegetarian foods, dry foods, snack foods, condiments, desserts, soups, baby formulas, and beverages representing the different levels of processing (**Table 1**), were chosen from local supermarkets.

Soybean Reference Material. As reference material for qualitative and quantitative analysis, Roundup Ready soybean (variety S14M7) was obtained from a Canadian seed distributor. Because the purity of the seed lot was unknown, single-seed extractions were done to confirm that individual seeds were transgenic. One of these DNA extractions was then used as reference material. The single-seed extract was diluted to 40 ng/μL and serially diluted to 12 pg/μL. Six of these dilutions (5 μL per reaction) were chosen to represent the standard curve encompassing 200 ng−60 pg of DNA.

Quantitative PCR Control. Soybean powder certified reference material (CRM) IRRM-410S (Fluka) 5% Roundup Ready soy used for quantitative real-time PCR was purchased from Sigma (Canada). A processed soy food sample, meat pâté, from the Genetically Modified Material Analysis (GeMMA), Scheme, Report GeM18, with an assigned RR soy content of 8.5%, was also used as a control.

Extraction of Genomic DNA. Each soy food product was homogenized in a Waring electric blender and then ground using an ice-cold mortar and pestle. Approximately 200 mg or 1 g samples from the ground material were used in the subsequent DNA extraction procedure. The single S14M7 soybean seed reference material (~200 mg) was ground using a chilled mortar and pestle, whereas 50 mg of the CRM soybean powder was extracted directly.

Modified Wizard. Briefly, 860 μL of extraction buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) sodium dodecyl sulfate], 100 μL of (5 M) guanidine hydrochloride, and 40 μL of (20 mg/mL) proteinase K were added to 200 mg of each ground sample and vortexed to mix thoroughly. For 1 g samples, the extraction buffer, guanidine hydrocloride, and proteinase K were scaled up proportionally. The samples were incubated at 60 °C for 30−60 min followed by a further incubation at 75 °C for 20 min to inactivate the proteinase K. The supernatant generated after centrifugation at full speed for 20 min (to pellet debris) was incubated for 10 min at 60 °C with RNase A added to a final concentration of 1 mg/mL. Following a 3 min centrifugation at full speed, 1 mL of the supernatant was mixed with 1 mL of Wizard resin (Promega) and then applied to a Wizard mini column using a 3 or 5 mL capacity disposable Luer-Lok syringe. For samples that had a dark supernatant (roasted soy nuts, biscuit, natto, and simulated bacon bits), the column was washed with 1 mL of CQW wash buffer (Nucleospin Food Kit, Macherey-Nagel). All samples were washed with 2 mL of 80% ethanol and then centrifuged at full speed for 10 min followed by a 10 min incubation at 37 °C to evaporate residual ethanol. The nucleic acids were eluted in 100 μL of 70 °C 10 mM Tris, pH 8.0.

Qiagen Stool Kit. The soy sauce, chocolate pudding, and miso homogenized samples were dried at 37 °C for 24 h to remove excess moisture. To 1 g of dried sample, ASL buffer (supplied with the Stool Kit) was added until a consistency was reached such that the sample flowed freely in the tube (2−3 mL). The samples were vortexed until thoroughly homogenized. Following a 5 min incubation at 70 °C, the samples were centrifuged at full speed for 10 min to remove particulates. The supernatant was divided into multiple 2.0 mL microfuge tubes and 1 InhibitEX tablet added to each (1 tablet/1.2 mL supernatant). If the volume was <1.2 mL per tube, additional ASL buffer was added. The samples were vortexed to suspend the InhibitEX tablet and then incubated at room temperature for 1 min. The samples were centrifuged

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Table 2. Primers Used for Qualitative and Quantitative PCR

primer/probe	sequence 5′ — 3′	product size (bp)	ref
lectin qualitative			
GMO3(forward)	gcccctactccaccccccatcc	118	15
GMO4(reverse)	gcccatctgcaagcctttttgtg		
RR qualitative			
RR04(forward)	ccccaagttcctaaatcttcaagt	180	24
RR05(reverse)	tgcgggccggctgcttgca		
lectin quantitative			
LecLC–F(forward)	cctctactccaccccccatcca	114	25
LecLC-R(reverse)	ccatctgcaagcctttttgtg		
LecLCFITC-1 (probe)	ttgccagcttcgccgcttc-FITC		
LecLCR705-1 (probe)	Red705-ttcaacttcacctttctatgccccgac		
RR quantitative			
RRLC-F20(forward)	accgtcttcccgttaccttg	119	25
RRLC-R15(reverse)	gccgggcgtgttgag		
RRSoyaLCFITC-1 (probe)	gccgatggcctccgcaca-FITC		
RRSoyaLCR640-1 (probe)	Red640-gaagtccgccgtgctgctcg		

at full speed for 3 min to pellet the InhibitEX particles. The supernatants were pooled and then incubated for 10 min at 60 °C with 1 mg/mL final concentration of RNase A. Following centrifugation at full speed for 3 min, the pooled supernatants were treated with Qiagen Proteinase K solution and AL (supplied with the Stool Kit) buffer proportionally (for every 200 μL of supernatant, 15 and 200 μL of Proteinase K and AL buffer were added, respectively) according to the manufacturer's instructions. After incubation at 70 °C for 10 min, the amount of 95% ethanol was added according to the manufacturer's instructions. This entire mixture was then applied to the QIAamp spin column. The column was then washed, and the DNA was eluted with 100 μL of AE buffer according to the manufacturer's instructions.

Determination of Yield and Quality of Nucleic Acid Extracts. The nucleic acid extracts were measured using the Biochrom Ultrospec 2100 pro spectrophotometer in 200 mM NaOH (*15*). A conversion factor of 1 OD = 37 ng/μL was used to convert absorbance into concentration units. Sample purity was determined by measuring the A_{260}/A_{280} ratio. The extracts (100 ng−200 ng) were further analyzed by electrophoresis on an 0.8% agarose gel containing 0.1 μg/mL ethidium bromide.

Qualitative PCR Detection of Lectin and RR Sequences. PCR amplification was performed using 100−200 ng of DNA and 100−400 ng of DNA for the lectin and RR assays, respectively. Of the extracts that could not be accurately measured spectrophotometrically (cheesies and miso; $A_{260} < 0.1$), 10 μL of extract was used in the PCR reactions. A 25 μL reaction contained 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), $MgCl_2$ (2.8 mM lectin; 1.8 mM RR; 2.0 mM chloroplast tRNA), 0.2 mM dNTP, 0.5 μM of each primer, and 1.25 units of Taq polymerase. Sequences of primers (Qiagen) used are shown in **Table 2**. The GeneAmp 9700 (Applied Biosystems) thermocycler was used with the following cycling program for lectin: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s, and a final extension at 72 °C for 5 min. The program for the RR PCR was similar except that 40 cycles were used. Products were electrophoresed on a 2% (w/v) agarose gel containing 0.1 μg/mL ethidium bromide.

The limit of detection (LOD) for both the lectin and RR qualitative PCR was determined from serial dilutions of S14M7 soybean seed DNA extracted using the modified Wizard method. Dilutions ranging from 50 ng to 10 pg (lectin) and from 80 to 1 pg (RR) were tested in triplicate. The LOD was defined as the lowest dilution for which the PCR product of expected size was visible by agarose gel electrophoresis in all three replicates. The conversion of mass to genome copy number was based on the haploid genome mass (1C value) for *Glycine max*, which is 1.13 pg, obtained from the Plant DNA C-values Database (*26*).

Real-Time Quantitative PCR of the Lectin and RR Genes. Fluorescence resonance energy transfer (FRET) hybridization probes were used to quantify in the LightCycler (Roche) real-time PCR system. The primers and probes (IT Biochem) used for the lectin and RR assays and the cycling conditions are described by Dahinden et al. (*15*, *25*). The donor probes for both targets were labeled with fluorescein-5-isothiocyanate (FITC), and the acceptor probes for lectin and RR genes were labeled with R705 and R640, respectively. Sequences of primers and probes used are shown in **Table 2**. PCR was performed in glass capillary tubes supplied by Roche. A total reaction volume of 20 μL contained 5 μL (15−200 ng) of DNA template, 50 mM Tris, pH 8.3, 0.4 μM of each probe, 0.25 mM of each dNTP, 0.5 μM of each primer, 250 μg/μL BSA, and 1 unit of Fast Start Taq polymerase (Roche). The lectin reactions contained 4.0 mM $MgCl_2$, and the RR reactions contained 2.5 mM $MgCl_2$. A serial dilution of $>3 \times 10^3$ (200 ng−60 pg per reaction) of total genomic DNA extracted from the S14M7 single seed, amplified in separate reaction tubes using either the lectin primers/probes or RR primers/probes, was used to generated the calibration curves for lectin and RR, respectively. The standard curves were the regression of crossing point (Cp) versus log of the nanograms of standard in each reaction, where the crossing point is the cycle at which the reaction fluorescence increases above a baseline level defined by the software (see **Table 3**). Each food sample was run in triplicate for each target. For the initial run, three different template concentrations for each sample were analyzed to determine which gave a Cp that best fit within the standard curve. In the subsequent run, this concentration was analyzed in duplicate, and the mean of all three Cp values was used in the analysis. Data were analyzed using LightCycler Data Analysis software version 3.5.5 and the "second derivative" algorithm.

Determination of Percent RR Soy Relative to Total Soy DNA in Food Samples. Two standard curves were generated from seven replicates of the lectin and RR assays. The equations from the linear regressions of these curves were used to calculate the nanograms of lectin or RR DNA in the food samples on the basis of the mean of the crossing points. The percent RR soy was determined from the ratio of nanograms of RR divided by the nanograms of lectin multiplied by 100. Some foods required more template in the RR reaction compared to the lectin reaction in order to bring the crossing points within the range of the standard curve (4 × template, meat alternatives 1 and 2, soup mix; 8 × template, protein bars 1 and 2;× 20 × template, tofu and 5% Fluka CRM), and therefore the RR values had to be divided by these factors before the ratio to lectin could be made.

RESULTS

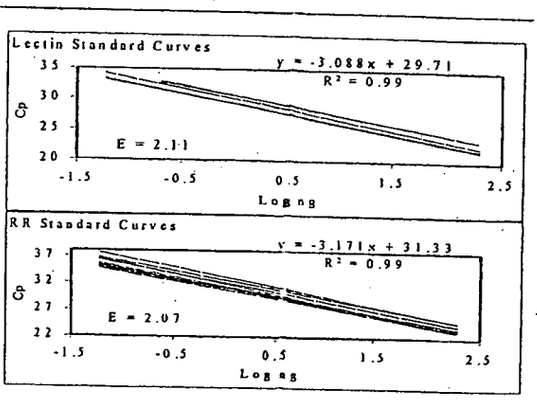
Classification of Soy-Containing Foods. The 39 foods chosen for this study had soy listed as an ingredient on the label and are representative of a wide range of soy-containing foods available in the Canadian marketplace. The soy ingredients included whole bean, flour, defatted flour, powder, soy protein, soy milk, and lecithin. The proportion of soy relative to other food ingredients varied from product to product and could not be experimentally determined. **Table 1** lists the soy foods and the degree of processing of the soy ingredients. Degree of processing was based on previously published descriptions of soybean products and their methods of preparation (*15*, *27*, *28*). Fermented, extruded (textured soy protein), pressed (lecithin and oil), and hydrolyzed soy protein soy ingredients were classified as highly processed. Mild alkaline extracted (isolated soy protein), water extracted (milk), precipitated (curd), and roasted ingredients were considered to be moderately processed. Whole beans and ground beans (flour) were considered to be the least processed. Foods with a number of different forms of soy in the ingredients were classified on the basis of the least processed form.

DNA Extraction. All samples were initially extracted using the modified Wizard method. Those samples that gave no or poor lectin amplification by qualitative PCR were re-extracted with the Qiagen Stool Kit (soy sauce, chocolate pudding, miso, gravy mix 1, natto, cracker 1, and cheesies). To determine the degree of degradation, and therefore the quality of the extracted DNA, ~200 ng of each sample was visualized by agarose gel electrophoresis (**Figure 1**). Many of the food extracts contained

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Table 3. Quantitative Results for the Soy Foods

Sample	Target	Standard (ng)	Cp[a]	SD[b]	ng Target	%RR/Soya	Corr. %RR/Soya
Standard	Lectin	200.00	22.53	0.74			
Standard	Lectin	100.00	23.61	0.77			
Standard	Lectin	25.00	25.35	0.65			
Standard	Lectin	6.25	27.17	0.58			
Standard	Lectin	0.78	30.27	0.72			
Standard	Lectin	0.06	33.36	0.32			
Standard	RR	200.00	23.92	0.67			
Standard	RR	100.00	25.06	0.62			
Standard	RR	25.00	26.79	0.71			
Standard	RR	6.25	28.82	0.83			
Standard	RR	0.78	31.92	0.88			
Standard	RR	0.06	35.02	0.39			
TVP	Lectin		25.58	0.10	21.7		
	RR		27.83	0.11	12.7	58	
Meat alternative 1	Lectin		24.77	0.06	39.8		
	RR[c]		33.14	0.08	0.067	0.17	<LOQ
Soup mix	Lectin		25.62	0.03	21.1		
	RR[c]		30.37	0.18	0.502	2.4	
Meat alternative 3	Lectin		26.53	0.08	10.7		
	RR[c]		33.80	0.29	0.042	0.39	5.0
Protein bar 1	Lectin		24.65	0.06	43.5		
	RR[d]		33.78	0.44	0.021	0.05	<LOQ
Protein bar 2	Lectin		25.16	0.11	29.7		
	RR[d]		34.61	0.33	0.012	0.04	<LOQ
Tofu	Lectin		25.39	0.09	25.1		
	RR[e]		33.87	0.38	0.008	0.03	0.43
Simulated bacon bits	Lectin		23.55	0.16	98.8		
	RR		25.85	0.03	53.5	54	
Frozen dessert	Lectin		26.44	0.20	11.5		
	RR		28.73	0.05	6.60	58	
Infant formula 1	Lectin		25.58	0.05	21.7		
	RR		27.73	0.13	13.7	63	
Bread 1	Lectin		28.30	0.37	2.86		
	RR		30.27	0.21	2.16	75	
Bread 2	Lectin		28.83	0.12	1.93		
	RR		30.81	0.18	1.46	76	2477
Biscuit	Lectin		26.41	0.10	11.7		
	RR		29.30	0.05	4.37	37	
Gravy mix 2	Lectin		27.60	0.11	4.82		
	RR		29.36	0.12	4.18	87	
5% Fluka	Lectin		25.30	0.05	26.8		
	RR[e]		26.92	0.06	1.23	4.6	
GeMMA Meat Pâté	Lectin		28.54	0.07	2.39		
	RR		33.85	0.18	0.16	6.7	



[a] Cp, crossing point (mean of nine replicates for standard curves and mean of three replicate for the food samples). [b] SD, standard deviation. [c] Four times more template used in the RR reaction compared to the lectin reaction. [d] Eight times more template used in the RR reaction compared to the lectin reaction. [e] Twenty times more template used in the RR reaction compared to the lectin reaction.

degraded DNA with an average fragment size of <564 bp. Yields from the various foods ranged from 0.15 μg/100 mg (soy sauce) to 38 μg/100 mg textured vegetable protein (TVP). The cheesies and miso samples extracted with the Qiagen Stool Kit both had absorbances at 260 nm of <0.1 and therefore could not be measured accurately. All of the samples had A_{260}/A_{280} ratios between 1.7 and 1.9 with the exception of soy sauce (2.3), cheesies (2.0), and coffee whitener (2.0).

Qualitative PCR. To determine if there was amplifiable soy DNA in the food extracts, PCR was performed using the lectin specific primers GMO3 and GMO4 (*15*). The LOD for the lectin assay was 50 pg (44 genome copies) of total soy DNA (data

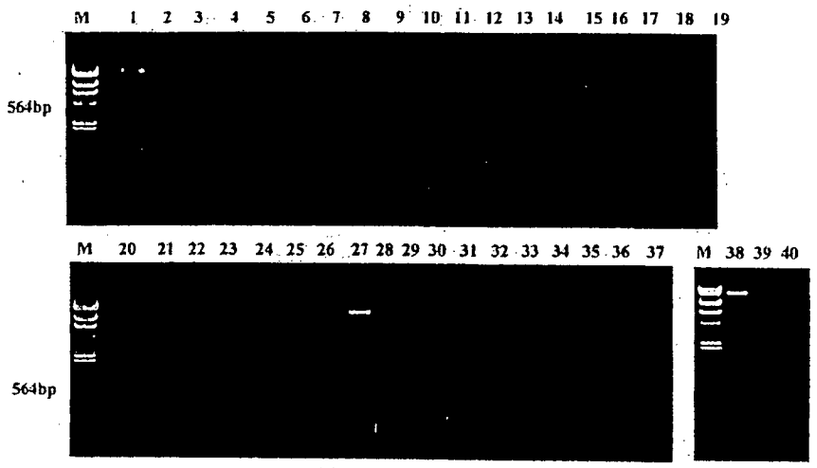


Figure 1. Agarose gel electrophoresis of nucleic acids extracted from the soy foods using the modified Wizard method unless otherwise stated: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) meat alternative 2; (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) coffee whitener; (lane 18) soy beverage; (lane 19) infant formula 2; (lane 20) miso (Qiagen Stool Kit); (lane 21) TVP; (lane 22) gravy mix 2; (lane 23) frozen dessert; (lane 24) soy nut snack; (lane 25) nutritional supplement bar; (lane 26) soup mix; (lane 27) gravy mix 1; (lane 28) boiled soybean; (lane 29) simulated bacon bits; (lane 30) crispbread; (lane 31) cracker 1 (Qiagen Stool Kit); (lane 32) cheese; (lane 33) cheesies (Qiagen Stool Kit); (lane 34) roasted soy nuts; (lane 35) natto; (lane 36) biscuit; (lane 37) flour; (lane 38) S14M7 soybean; (lane 39) soy sauce (Qiagen Stool Kit); (lane 40) chocolate pudding (Qiagen Stool Kit); (lane M) molecular weight marker II (Roche).

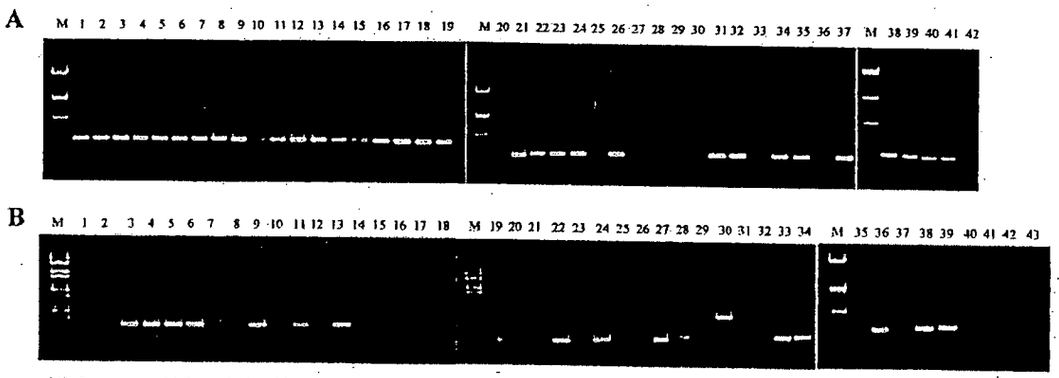


Figure 2. (A) Qualitative PCR analysis of lectin and Roundup Ready DNA in food samples. The 118 bp PCR product represents the amplified region of the lectin gene using primers GMO3 and GMO4: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) meat alternative 2; (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) coffee whitener; (lane 18) soy beverage; (lane 19) infant formula 2; (lane 20) miso; (lane 21) TVP; (lane 22) gravy mix 2; (lane 23) frozen dessert; (lane 24) soy nut snack; (lane 25) nutritional supplement bar; (lane 26) soup mix; (lane 27) gravy mix 1; (lane 28) cracker 1; (lane 29) boiled soybean; (lane 30) simulated bacon bits; (lane 31) natto; (lane 32) biscuit; (lane 33) chocolate pudding; (lane 34) soy sauce; (lane 35) positive control (S14M7); (lane 36) flour; (lane 37) cheesies; (lane 38) cheese; (lane 39) crispbread; (lane 40) roasted soy nuts; (lane 41) positive control (S14M7); (lane 42) water control (no DNA); (lane M) 50 bp DNA ladder (Invitrogen). (B) The 180 bp PCR product represents the CTP4–CP4EPSPS junction of the Roundup Ready transgene using primers RR04 and RR05: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) positive control (S14M7); (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) infant formula 2; (lane 18) cheesies; (lane 19) gravy mix 2; (lane 20) soy nut snack; (lane 21) nutritional supplement bar; (lane 22) soup mix; (lane 23) miso; (lane 24) cracker 1; (lane 25) TVP; (lane 26) meat alternative 2; (lane 27) water (no DNA); (lane 28) coffee whitener; (lane 29) soy beverage; (lane 30) cheese; (lane 31) crispbread; (lane 32) roasted soy nuts; (lane 33) gravy mix 1; (lane 34) frozen dessert; (lane M) molecular weight marker VI (Roche); (lane 35) boiled soybeans; (lane 36) simulated bacon bits; (lane 37) natto; (lane 38) biscuits; (lane 39) positive control (S14M7); (lane 40) flour; (lane 41) chocolate pudding; (lane 42) soy sauce; (lane 43) water (no DNA); (lane M) 50 bp ladder (Invitrogen).

not shown). Figure 2A shows the amplified lectin band (118 bp) for each of the soy foods. Lectin PCR products from miso, gravy mix 1, cheesies, and natto were weak, and there were no clear amplification products of the expected size from the

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cracker 1 sample. These foods were re-extracted using the Qiagen Stool Kit, which improved amplification from cheesies, miso, and cracker 1 but not from natto or gravy mix 1 (**Figure 2A**).

The 39 food samples were subsequently analyzed using primers RR04 and RR05 (15) specific for the CTP4–CP4EPSPS junction of the RR transgene. Of these 39 samples, 28 tested positive for RR (**Figure 2B**). The LOD for this assay was 10 pg (eight genome copies) of RR soy DNA (data not shown). Three samples, gravy mix 1, miso, and cheesies, which amplified poorly with the lectin-specific primers, weakly amplified with the RR specific primers. RR could not be detected in natto and cracker 1, which were also only weakly amplified with the lectin primers. The dried (organic) soybean, soybean pâté, soup, meal replacement beverage, soy nut snack, nutritional supplement bar, crispbread, chocolate pudding, and soy sauce, which strongly amplified with the lectin primers, also tested negative in the RR PCR. Bands with a higher molecular weight than expected were observed in miso, cracker 1, coffee whitener, and crispbread. In previous experiments, we found that amplification from rye and wheat using the RR-specific primers gave amplified products of sizes similar to those in the crispbread and cracker 1, respectively, suggesting nonspecific amplification from rye or wheat in these samples (unpublished results).

Quantitative PCR. The 28 food samples that tested positive for the RR trait were further analyzed by quantitative real-time PCR. The standard curves for both the lectin and the RR assays were made from serially diluted S14M7 DNA (see Material and Methods). Dilutions in the range of 200–0.06 ng were used to generate the standard curves. Nine interassays for lectin and RR were performed, and the mean efficiencies (E) were calculated to be 2.11 and 2.07, respectively (**Table 3**). Data were linear through the range of dilutions; therefore, 0.06 ng (53 genome copies) was defined as the limit of quantification (LOQ). This limit is well above the experimentally determined LOQ of 30 copies reported by Berdal and Holst-Jensen (*4*, *15*). For a pure (100%) soy product, the LOQ expressed as %RR/soy can be defined as the lowest quantifiable amount of RR soy DNA (0.06 ng) divided by the highest quantifiable amount of soy DNA (200 ng) multiplied by 100 (e.g., 0.06 ng/200 ng × 100 = 0.03%). The LODs for the lectin and RR quantitative assays were not determined. The total soy DNA in the foods was determined using primers LecLC-F and LecLC-R and probes LecLCFITC-1 and LecLCR705-1, and the total RR DNA in the food products was determined using primers RRLC-F20 and RRLC-R15 and probes RRSoyLCFITC-1 and RRSoyLC640-1 (**Table 2**).

Initially, three serial dilutions of the DNA food extracts (25–200 ng) were analyzed to determine E for both the lectin and RR assays. A subsequent assay in duplicate for each food extract was used to calculate the %RR DNA relative to total soy DNA. **Table 3** summarizes the results for the foods that were above the LOQ for the RR quantitative PCR assay. The nanograms of target DNA was determined by comparing the mean crossing point of each sample to the standard curve. Cheesies, miso, and gravy mix 1 had a weak lectin amplicon in the qualitative PCR (**Figure 2A**) and were below the limit of quantification for the lectin in the real-time PCR (data not shown). For this reason, the amount of RR soy in these samples was not quantified. Fourteen of the 36 food samples contained quantifiable levels of RR soy that ranged from 0.03 to 87%. Foods that contained levels of RR soy >5% included TVP, frozen dessert, infant formula 1, simulated bacon bits, bread 1, bread 2, biscuit, and gravy mix 2. Foods that contained <5% RR soy included meat alternative 1, meat alternative 3, tofu, protein bar 1, protein bar 2, and soup mix. In order for the quantitative results for these foods to fall within the RR standard curve, 4–20 times more DNA had to be added to the RR reactions compared to the lectin reactions. Eleven samples contained only trace amounts of amplifiable RR-specific DNA, which could not be quantified in the real-time PCR assays. These included yogurt, boiled soybean, flour, cheese, infant formula 2, soy beverage, roasted soy nuts, cracker 2, coffee whitener, meat alternative 2, and soy nut spread.

Table 4. Lectin and RR PCR Efficiencies for the Soy Foods

food	lectin E[a]	ΔE[b]	RR E[c]	ΔE[d]	$\Delta\Delta E$[e]
TVP	1.95	0.16	1.85	0.22	0.06
meat alternative 1	2.11	0.00	2.31	−0.24	0.24
soup mix	1.93	0.18	1.81	0.26	0.08
meat alternative 3	2.05	0.06	1.88	0.19	0.13
protein bar 1	1.95	0.16	2.18	−0.11	0.27
protein bar 2	1.90	0.21	2.13	−0.06	0.27
tofu	1.98	0.13	1.83	0.24	0.11
simulated bacon bits	2.08	0.03	1.94	0.13	0.10
frozen dessert	1.81	0.30	1.77	0.30	0.00
infant formula 1	2.01	0.10	1.96	0.11	0.01
bread 1	2.01	0.10	2.05	0.02	0.08
bread 2	1.98	0.13	1.74	0.33	0.20
biscuit	1.97	0.14	1.97	0.10	0.04
gravy mix 2	1.95	0.16	1.86	0.21	0.05
5% Fluka	1.97	0.14	1.95	0.12	0.02

[a] E, efficiency of the lectin PCR calculated from the slope of logarithm of the ng of template versus Cp for three dilutions of the sample ($E = 10^{(-1/\text{slope})}$). [b] ΔE, difference in efficiency between the food sample and the lectin standard curve (2.11, calculated from the slope in **Table 3**). [c] E, efficiency of the RR PCR for three dilutions of the sample. [d] ΔE, difference in efficiency between the food sample and the RR standard curve (2.07, calculated from the slope in **Table 3**). [e] $\Delta\Delta E$, difference in efficiency of the food samples between the lectin and RR PCR.

A positive control containing 5% RR soy (Fluka) was analyzed together with the samples and calculated to contain 4.6% RR/total soy DNA. GeMMA sample GeM18, meat pâté, was used as a processed food control. GEM18 was given an assigned value of 8.5% RR soy by GeMMA and was calculated in this study to contain 6.7% RR soy.

Calculations of %RR soy in the food samples were based on the assumption that the real-time PCR amplification E values of the standards and samples were the same. To determine the validity of this assumption, E values of the food samples were calculated and compared to the standards. Whereas E values for the standards are based on a standard curve consisting of six dilutions spanning a little more than 3 orders of magnitude (200–0.06ng) repeated nine times for a total of 54 data points, each food sample consisted of only three dilutions spanning <1 order of magnitude repeated three times for a total of nine data points. The amplification efficiencies of the food samples ranged from $1.81 \leq E \leq 2.11$ for lectin and from $1.74 \leq E \leq 2.31$ for RR (**Table 4**). For the lectin reaction, all samples had E values equal to or less than the standard. For the RR assay, meat alternative 1, protein bar 1, and protein bar 2 had E values greater than the standard ($E = 2.31$, 2.18, and 2.13) with the other food samples having E values equal to or less than the standard (**Table 4**). The difference in E between food sample and standard for each of the lectin and RR assays is expressed as ΔE. For most of the foods, ΔE values for the RR assay deviated from the standard to a greater degree than the lectin assay. The difference in ΔE values between the lectin and RR assays for each food sample is expressed as $\Delta\Delta E$. A low value of $\Delta\Delta E$ indicates that the efficiencies for the lectin and RR

assays for a sample deviated from the standard to the same degree. Eight food samples and the 5% Fluka standard had $\Delta\Delta E$ values of ≤ 0.1. The remaining six foods had $\Delta\Delta E$ values >0.1 (Table 4). For food samples with $\Delta\Delta E$ values >0.1, a correction factor (*29*)

$$Cp_{corr} = Cp_{meas} \times \frac{\log (E_f)}{\log (E_s)} \quad (1)$$

where E_f is the efficiency measured for the food sample and E_s is the efficiency for the corresponding standard, was used to calculate corrected percent RR values (**Table 3**). When this correction factor was applied, meat alternative 1 and protein bars 1 and 2 were no longer within the LOQ, for bread 2 the %RR values rose from 76 to >2400%, and for one sample (meat alternative 3) the %RR soy changed significantly (from 0.39 to 5.0%) which could alter the classification of this food with respect to a proposed Canadian labeling threshold of 5%.

DISCUSSION

In anticipation of food labeling for genetically modified products in Canada, a pilot project was undertaken to detect and quantify RR soy in a variety of soy-containing foods available in the Canadian marketplace. RR soy was chosen because the transgenes for this event are well characterized, a number of published tests for the detection and quantification are available, and it is widely grown and used in both Canada and the United States and was therefore expected to be detectable in a wide range of food products. From the survey, soy could be detected in 100% of the 39 food samples tested, and RR soy was detected in 28 (72%) of these foods. Of the foods testing positive, 11 foods (28%) contained quantifiable levels of RR soy, 8 of which (21%) contained levels of RR soy >5% and would be candidates for labeling.

Assay Controls. To test the accuracy of quantitative tests, two known controls were analyzed. The 5% RR soybean powder (Fluka) IRMM certified reference standard was calculated to contain 4.6% RR soy, an error of only 8%. The second control, GeM18 meat pâté sample, is more representative of the processed foods analyzed in this study. To summarize, the composition and preparation of this sample was as follows. A 6−7% RR soy (w/w) of total soy flour mix was added to other pâté ingredients (pig liver, pork belly, water, skimmed milk powder, salt, sorbic acid, sodium polyphosphate, pepper, onion powder, and monosodium glutamate) at a level of 5%, mixed, and baked at 130 °C for 1 h and then at 150 °C until a core temperature of 80 °C was obtained. Homogeneity testing of the pâté samples by GeMMA (10 subsamples, 2 replicates of each) gave a mean RR soy/total soy value of 5.5% with a range from 3.8 to 8.9%. The assigned %RR value for the pâté, based on the consensus mean of 81 laboratories participating in the GeM18 proficiency panel, was 8.5%, with acceptable individual results (based on z scores) ranging from 3.4 to 21.4%. In this study, the meat pâté sample was calculated to contain 6.7% RR soy/total soy, which is close to both the mean value determined by homogeneity testing and the assigned value. Close agreement of the experimentally determined and accepted values for both the RR flour and processed food controls gives confidence that the values obtained for the unknown processed food samples are accurate.

Food-Processing Effects. The food samples tested contained soy as an ingredient ranging from low (whole bean or flour) to highly processed (fermented bean). In general, it can be assumed that increasing levels of processing result in a decrease in the amount and quality of soy DNA that can be extracted due to degradation of the DNA caused by the different processing methods. This can have a significant effect on the ability to detect/quantify the amount of soy in these foods by PCR. Agarose gel electrophoresis of the DNA extracted from the food samples showed that many contained highly degraded DNA with average fragment sizes of <564 base pairs (bp). The four separate PCR assays used in this study amplified fragments from 114 to 180 bp in length, which are small enough to amplify from DNA that has been significantly degraded. For two of the most highly processed food products tested, miso and cheesies, both lectin and RR could be detected but not quantified. This is due to the very low yield of DNA obtained, which could not be quantified spectrophotometrically. To obtain sufficient DNA for quantitative analysis, larger amounts of food sample would have to be used in the DNA extraction procedure. For both miso and cheesies this would likely have resulted in relatively high levels of RR because both could be detected in the small amount of DNA extracted. However, it should be noted that the LOD of the RR PCR is lower than the lectin PCR (8 copies versus 44 copies).

Extraction Efficiencies and DNA Purification. Previous studies have reported difficulty extracting amplifiable DNA from soy sauce (*6*, *15*, *21*); however, using 1 g of dried soy sauce and the Qiagen Stool Kit, enough DNA could be obtained for spectrophotometric quantification and subsequent amplification. DNA from food products containing chocolate have been shown to require additional purification steps to remove the cocoa plant secondary metabolites thought to inhibit PCR (*1*, *15*, *20*−*22*). Although the lectin-specific PCR did not produce a product from the Wizard extracted chocolate pudding, DNA obtained from chocolate pudding using the Qiagen Stool Kit could be amplified. The Qiagen Stool Kit was designed to effectively remove known inhibitors of PCR and has been demonstrated by Tengel et al. (*22*) to be effective in removing PCR-inhibitory compounds in cocoa products. However, for protein bars 1 and 2, which list cocoa as an ingredient, DNA extracted using the modified Wizard method could be amplified in the PCR assays. The proportion of cocoa in the protein bars may be low enough that the PCR-inhibitory compounds are not enough to significantly affect the PCR. The Qiagen Stool Kit also improved the quantitative PCR from cheesies, miso, and cracker, although the amount of DNA obtained was too low to be accurately measured spectrophotometrically for cheesies and miso. Lectin could be weakly detected in gravy mix 1, natto, and cracker 1, and RR could be detected in only gravy mix 1. Soy in the form of oil/hsp in gravy mix 1 likely contains only low amounts of degraded soy DNA, which does not correspond to the high molecular weight DNA extracted from gravy mix 1 (**Figure 1**). The majority of DNA from these samples probably originates from ingredients other than soy. Alternatively, these extracts may contain PCR inhibitors not removed using the Qiagen Stool Kit. Infant formulas 1 and 2 contained the same type of soy ingredients, soy milk, isp, and oil, and both showed comparable DNA fragment sizes on the agarose gel (**Figure 1**). Infant formula 1 contained 63% RR soy, whereas infant formula 2, which contained about half as much total soy as infant formula 1, was below the LOQ for the RR assay. It would therefore appear that very little of the soy in infant formula 2 was RR soy, whereas in infant formula 1 a high percentage of the soy was derived from RR soy. There is a large difference in the quality of DNA in the extracts among the foods that were shown to contain higher levels of RR soy. For example, the DNA extracted from the frozen dessert is so degraded that it is not

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visible on the agarose gel, whereas breads 1 and 2 have a greater abundance of higher molecular weight DNA fragments (**Figure 1**).

These results highlight problems of accurately measuring the amount of soy that is highly processed and/or present in a complex food product. In the first situation, highly processed soy products require a very large sample in order to obtain sufficient DNA for analysis. In the second situation, where soy is an ingredient of a complex food product, the total amount of DNA extracted may not be limiting, but the quality and quantity of the DNA to be amplified are limiting. If the food contains soy as a highly processed ingredient together with other food ingredients, these two factors are compounded. This has significant implications for compliance labeling purposes. It has been suggested that certain processed food ingredients that contain little or no amplifiable DNA be exempt from testing/labeling (*30*). However, this raises additional questions on how products to be exempt are to be defined. An alternative solution would be to test for genetically modified soy before processing or before it is added as a food ingredient.

Amplification Efficiencies. For precise quantification by real-time PCR, amplification efficiencies of the standards and sample must be the same (*4*, *15*). For many quantitative PCR experiments this assumption is made but not tested. When the source material for the standards and the samples are of the same matrix (e.g., both source and sample DNA are extracted from whole soybean), this assumption is probably close enough to be valid. Calculations show that differences in PCR efficiencies of only 5% can result in a 2-fold difference in measurement after 26 cycles (*31*). In this study, the standards consisted of high molecular weight DNA extracted from unprocessed soybean. The only current certified reference standards available on the market for quantitative PCR analysis of RR soy are ground flour mixtures produced by the Joint Research Centre, Institute for Reference Materials and Measurement. In contrast, many of the food samples analyzed in this study contained degraded soy DNA as a result of processing. In addition, soy was often only one of several food ingredients in a complex food sample. Essentially, each food represents a different matrix, often very different from the unprocessed soybean standard, used in this study. Each food potentially contains compounds that are not removed during the DNA extraction procedure, which could affect the efficiency of the PCR despite the use of DNA extraction procedures that can remove known PCR inhibitors. Therefore, for the quantitative PCR analysis of foods for genetically modified ingredients, the assumption cannot be made that efficiencies of standards and samples are the same.

Several methods have been reported to compare differences in PCR efficiencies (E) between standard and sample. A series of multiplex reactions can be used to calculate E (*32*); E values can be determined on the basis of the kinetics of the individual sample reactions (*33*) or calculated on the basis of a series of dilutions (*29*). In this study, E values were based on a series of dilutions. The mean calculated values for the standards were $E = 2.11$ (lectin) and $E = 2.07$ (RR). Each value is based on a total of 54 data points spanning a dilution range of 3×10^3, which is statistically twice the number required for an accurate determination of E over this range. E values for the food samples, however, are based on only nine data points for over a dilution range of <1 order of magnitude. As the dilution range decreases, the number of data points that are required for an accurate determination of E increases. Whereas a dilution range of 10^3 requires only 27 data points, a dilution range of 10^1 would require 240 data points, which is clearly not practical. It is also not possible to significantly expand the dilution range by further diluting the sample due to the limited amount of soy DNA extracted from the foods or by increasing the total amount of DNA used in the assay due to inhibitory effects. As a result, calculated E values for the foods are an approximation only and most likely overestimate the difference in E values between the standards and foods. E values for the foods differed from the standards (ΔE) by as much as 0.30 (lectin) and 0.33 (RR). In most cases, E values for the food samples were less than that of the standard. This suggests that inhibitors were present in the food DNA extractions. If the reduction in E of the sample was due to the presence of inhibitors, it might be expected that both the lectin and RR assays would be equally affected. This can be determined from the difference in the ΔE values of the lectin and RR assays ($\Delta\Delta E$). A small $\Delta\Delta E$ for a food sample indicates that the E values for both the lectin and RR assays differed from the E values of the standard by the same amount. Eight foods and the 5% Fluka standard had $\Delta\Delta E$ values of ≤ 0.1. For example, in the frozen desert sample, Cp values for lectin and RR are 26.44 and 28.73 and ΔE values were 0.30 and 0.30, respectively. For both reactions this would result in a calculated underestimation of the amount of lectin and RR by ~80×. However, the $\Delta\Delta E$ value is 0.00. Therefore, because the efficiencies of the lectin and RR assays deviate from the mean value of the standards by the same degree, the ratio of the measured amounts of each (percent RR) is still valid, even if the absolute values are incorrect. Some of the foods had relatively high $\Delta\Delta E$ values (>0.10) which could indicate that the %RR determined is incorrect. High $\Delta\Delta E$ could be due to a number of factors, for example, differences in the robustness of the lectin and RR assays. A greater variation in the RR assay for the standards and the food samples was observed compared to the lectin assay, which could indicate that the RR assay is less robust. However, greater variations in the food quantitative RR assay may also be because the amount of RR relative to the total amount of soy can be much lower. Meat alternative 1, protein bar 1, and protein bar 2 contained low measured amounts of RR (0.04−0.39%) and had the largest $\Delta\Delta E$ values (0.24−0.27). To compensate for foods with $\Delta\Delta E$ values of >0.10, a correction factor was applied (eq 1). When this correction factor was applied to meat alternative 1 and protein bars 1 and 2, the amount of RR present was calculated to be below the LOQ. It has been demonstrated that low concentrations of target can lead to a calculation artifact with the LightCycler (*34*). The curve of Cp versus log of concentration, at low target concentrations, is no longer linear but parabolic, and calculations of E within this range would give artificially high values if a correction factor was not used. This appears to be the case for meat alternative 1 and protein bars 1 and 2.

Applying the correction factor to bread 2 ($\Delta\Delta E = 0.20$) resulted in an increase in %RR from 76 to 2477%, which clearly is not possible. This result is probably due to an error in the calculation of E for bread 2 due to an insufficient number of data points. Bread 2 contained the least amount of soy of all foods quantified (<1% of the total DNA extracted could be attributed to soy); slight errors in measurement at these concentrations would have a large effect on calculated E values. The correction factor was also applied to meat alternative 3 ($\Delta\Delta E = 0.13$) and tofu ($\Delta\Delta E = 0.11$), resulting in a measured increase in %RR of ~10× from 0.39 to 5.0% and from 0.03 to 0.43%, respectively. For all corrected samples, the change in calculated %RR would not have affected the classification and subsequent labeling under the newly proposed Canadian voluntary labeling guide for genetically modified products. The

usefulness of the correction factor is directly related to the accuracy with which E for the foods can be determined. Unfortunately, there is not a fully satisfactory and practical method for determining E for the foods. The method used here gives only an approximation that probably overestimates the change in E relative to the standards. As such it presently could be useful in determining the amount of error in the final percent values until more accurate methods of determining E for the foods have been developed.

Sampling Issues. The food samples tested were individual items that represent a random sampling. The results obtained apply only to those items, and no statement about any particular product can be inferred with respect to the presence or amount of RR soy. For labeling purposes, it would be necessary to test a population of samples from any one batch of products in order to make a meaningful statistical statement concerning the level of RR soy present. What has been demonstrated is a practical method for the detection and quantification of RR soy in a wide range of real world food products that have undergone various degrees of processing. Both qualitative and quantitative PCR methods are strongly influenced by the amount, quality, and presence of inhibitors in the DNA extractions from these foods. In some cases, the amount/quality of DNA extracted was too low for quantification either because the soy was highly processed and the DNA degraded or because the amount of soy present in the food was too low. Factors that alter the efficiencies of the quantitative PCRs unequally can dramatically affect the end results; however, it is possible, in theory, to compensate using a correction factor. Further work needs to be done to test whether the use of a correction factor is valid when applied to food samples.

Summary. For the majority of foods tested, unequivocal results could be obtained for the presence/absence of soy and RR soy. In the quantitative assays, two main groups were observed: foods with trace amounts of RR soy, $\leq 0.4\%$, suggesting adventitious contamination of soy with RR soy, and foods with relatively high levels of RR soy, $\geq 37\%$, suggesting RR soy was an intended ingredient. Of the 39 foods tested, only 2 samples were close to the proposed 5% level for labeling (soup mix and meat alternative 3). Many questions remain to be answered before reliable routine testing of foods can take place. For example, what is the accuracy and how much error is in the calculated %RR when testing foods. Although most of the foods tested fell into two broad groups that contained either trace amounts of RR soy or relatively high amounts of RR soy and could be easily separated as either above or below 5%, categorizing foods that test close to this level is more problematic until there is a better understanding of the amount of error present in the calculation. In particular, methods need to be developed for a more accurate determination of E for the foods being tested so that the correction factor can be more confidently applied. Failing that, an alternative would be to develop reference standards that are more representative of the foods being tested. These questions are difficult to answer without knowing the true %RR in the food samples, and studies using defined nontransgenic/transgenic flour mixtures are not truly representative of real-world food samples. Therefore, further real-time quantitative PCR experiments using defined flour mixtures processed into foods will be necessary.

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JF030803G

Application for authorization to place on the market MON 89788 soybean in the European Union, according to Regulation (EC) No 1829/2003 on genetically modified food and feed

Part II

Summary

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A. GENERAL INFORMATION

1. Details of application

a) Member State of application The Netherlands.
b) Notification number Not available at the time of submission.
c) Name of the product (commercial and other names) The name of the product is Roundup RReady2Yield™ soybean. The Monsanto development code for this genetically modified soybean is MON 89788. MON 89788 varieties will be marketed under the name of the variety, in association with the trademark Roundup RReady2Yield™ soybean.
d) Date of acknowledgement of notification Not available at the time of submission.

2. Applicant

a) Name of applicant	
Monsanto Company, represented by Monsanto Europe S.A.	
b) Address of applicant	
Monsanto Europe S.A.	Monsanto Company
Avenue de Tervuren 270-272	800 N. Lindbergh Boulevard
B-1150 Brussels	St. Louis, Missouri 63167
BELGIUM	U.S.A.
c) Name and address of the person established in the Community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor, if different from the applicant (Commission Decision 2004/204/EC Art 3(a)(ii)) MON 89788 will be produced in other world areas and will be imported and used in the European Union by operators that have traditionally been involved in the commerce, transport, processing and use of soybean and soybean-derived products in the EU.	

™ Roundup RReady2Yield is a trademark of Monsanto Technology LLC.

3. **Scope of the application**

(x) **GM plants for food use**

(x) **Food containing or consisting of GM plants**

(x) **Food produced from GM plants or containing ingredients produced from GM plants**

(x) **GM plants for feed use**

(x) **Feed containing or consisting of GM plants**

(x) **Feed produced from GM plants**

(x) **Import and processing (Part C of Directive 2001/18/EC)**

( ) **Seeds and plant propagating material for cultivation in Europe (Part C of Directive 2001/18/EC)**

4. **Is the product being simultaneously notified within the framework of another regulation (e.g. Seed legislation)?**

Yes ( )	No (x)
If *yes*, specify	

5. **Has the GM plant been notified under Part B of Directive 2001/18/EC and/or Directive 90/220/EEC?**

Yes ( )	No (x)
If *no*, refer to risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC The protein expression, the composition, the safety, the agronomic and the phenotypic characteristics of MON 89788 have been studied at multiple locations in North and South America that cover a range of environmental conditions. The data collected from these field releases have been used in the risk assessment presented in the MON 89788 application. A summary of the conclusions of the risk analysis that demonstrate the safety of MON 89788 to humans, animals and to the environment, have been presented in the respective sections throughout this summary.	

6. **Has the GM plant or derived products been previously notified for marketing in the Community under Part C of Directive 2001/18/EC or Regulation (EC) 258/97?**

Yes ( )	No (x)
If *yes*, specify	

BNSDOCID: <XP_____2411357A__I_>

7. **Has the product been notified in a third country either previously or simultaneously?**

Yes (x)	No ( )

If *yes*, specify

MON 89788 has been notified in the U.S.A. for the full range of uses as traditional soybean, including the cultivation of varieties. In addition, applications for the import and use of MON 89788 are currently under review in other countries around the world. The scope and the status of these pending regulatory reviews typically depend on the country and its local regulatory framework.

8. **General description of the product**

a) **Name of the recipient or parental plant and the intended function of the genetic modification**

The first genetically modified soybean authorized for import and use in the EU was Roundup Ready® soybean 40-3-2. Roundup Ready soybean 40-3-2 was developed by Monsanto Company by incorporating the *cp4 epsps* coding sequence derived from the common soil bacterium *Agrobacterium* sp. strain CP4 into the soybean genome using the particle acceleration method. Roundup Ready soybean 40-3-2 produces the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) that is less sensitive to inhibition by glyphosate[1] compared to plant endogenous EPSPS. The CP4 EPSPS protein renders Roundup Ready soybean 40-3-2 tolerant to glyphosate, which is the active ingredient of Roundup® herbicides. The utilization of Roundup herbicides plus Roundup Ready soybean 40-3-2, collectively referred to as the Roundup Ready soybean system, has provided significant convenience in weed control, encouraged the use of conservation-tillage and provided positive economic impact to the farmers. In 2005, Roundup Ready soybean 40-3-2 was planted on approximately 87% of the U.S.A. and 60% of the global soybean areas, which makes it the most cultivated biotechnology product to date.

Developments in biotechnology and molecular-assisted breeding have enabled Monsanto to generate a second-generation glyphosate-tolerant soybean product, Roundup RReady2Yield™ or MON 89788. MON 89788 produces the same CP4 EPSPS protein as Roundup Ready soybean 40-3-2. MON 89788 was produced by introducing the *cp4 epsps* gene cassette driven by a chimeric constitutive promoter into the soybean genome. The transformation was based on a new technique of *Agrobacterium*-mediated gene delivery to the soybean meristem, where cells were induced directly to form shoots and give rise to genetically modified plants. This technique allowed the direct transformation of the

® Roundup and Roundup Ready are registered trademarks of Monsanto Technology LLC.
[1] N-phosphonomethyl-glycine is the active ingredient in the non-selective, foliar-applied, broad-spectrum, post-emergent Roundup herbicides.

cp4 epsps gene cassette into elite soybean germplasm such as the Asgrow soybean variety A3244, which is known for its superior agronomic characteristics and high yielding property. Using elite germplasm as the base genetics, the superior agronomic characteristic of A3244 can be introgressed to other soybean varieties through crosses with MON 89788. In general, MON 89788 has been found to have a 4 to 7% yield advantage compared to Roundup Ready soybean 40-3-2 in the same elite genetic background (A3244). MON 89788 will continue to provide growers flexibility, simplicity and cost effective weed control options of the Roundup Ready soybean system. In addition, MON 89788 and the varieties containing this trait have the potential to enhance yield and thereby further benefit farmers and the soybean industry.

b) Types of products planned to be placed on the market according to the authorisation applied for

This application is for authorization of MON 89788 for import, food and feed use according to Articles 5 and 17 of Regulation (EC) No 1829/2003 on genetically modified food and feed. The scope of this application does not include the cultivation of MON 89788 varieties in the EU.

c) Intended use of the product and types of users

MON 89788 soybean will be used in the EU in the same manner as current commercial soybean and by the same operators currently involved in the trade and use of soybean.

d) Specific instructions and/or recommendations for use, storage and handling, including mandatory restrictions proposed as a condition of the authorisation applied for

MON 89788 is substantially equivalent to traditional soybean, except for its tolerance to glyphosate, which is a trait of agronomic interest. This soybean was shown to be as safe and nutritious as traditional soybean. Therefore, MON 89788 and its derived products will be stored, packaged, transported, used and handled in the same manner as current commercial soybean. No specific conditions are warranted or required for the food and feed use of MON 89788.

e) Any proposed packaging requirements

MON 89788 is substantially equivalent to traditional soybean, except for its tolerance to glyphosate. Therefore, MON 89788 and derived products will be used in the same manner as other soybean and no specific packaging is required. (For labelling, *see* question 8.f).

BNSDOCID: <XP_____2411357A__I_>

f) **A proposal for labelling in accordance with Articles 13 and 25 of Regulation (EC) 1829/2003. In the case of GMOs, food and/or feed containing, consisting of GMOs, a proposal for labelling has to be included complying with the requirements of Article 4, B(6) of Regulation (EC) 1830/2003 and Annex IV of Directive 2001/18/EC.**

In accordance with Regulations (EC) No 1829/2003 and 1830/2003, the current labelling threshold of 0.9% will continue to be applied for the marketing of MON 89788 and derived products.

Operators shall be required to label products containing or consisting of MON 89788 with the words "genetically modified soybean" or "contains genetically modified soybean" and shall continue to declare the unique identifier MON-89788-1 in the list of GMOs that have been used to constitute a mixture that contains or consists of this GMO.

Operators shall be required to label foods and feeds derived from MON 89788 with the words "produced from genetically modified soybean". In the case of products for which no list of ingredients exists, operators shall continue to ensure that an indication that the food or feed product is produced from GMOs is transmitted in writing to the operator receiving the product.

Operators handling or using MON 89788 and derived foods and feeds in the EU shall be required to be aware of the legal obligations regarding traceability and labelling of these products. Given that explicit requirements for the traceability and labelling of GMOs and derived foods and feeds are laid down in Regulations (EC) No 1829/2003 and 1830/2003, and that authorized foods and feeds shall be entered in the Community Register, operators in the food/feed chain will be fully aware of the traceability and labelling requirements for MON 89788. Therefore, no further specific measures are to be taken by the applicant.

g) **Unique identifier for the GM plant (Regulation (EC) 65/2004; does not apply to applications concerning only food and feed produced from GM plants, or containing ingredients produced from GM plants)**

MON-89788-1

h) **If applicable, geographical areas within the EU to which the product is intended to be confined under the terms of the authorisation applied for. Any type of environment to which the product is unsuited**

MON 89788 is suitable for use throughout the EU.

9. Measures suggested by the applicant to take in case of unintended release or misuse as well as measures for disposal and treatment

Because this application is for consent to import and use MON 89788 as any other soybean, not including the cultivation of varieties of MON 89788 in the EU, environmental release would be more likely to occur during import, storage and processing of MON 89788. However, modern methods of soybean handling minimize losses of seed, so there is little chance of germination of spilt soybeans resulting in the development of mature MON 89788 plants in the EU. Moreover, in the event of incidental spillage, the establishment of volunteer plants would be unlikely, since soybean cannot survive without human assistance and is not capable of surviving as a weed due to selection over centuries of cultivation. Soybean is not documented as a source of volunteer plants in rotational crops, which results from the combination of absence of seed dormancy, poor seed survivability in soils, frost sensitivity of soybean seedlings and soil preparations prior to the planting of a subsequent crop (which includes destruction of any existing vegetation and soil cultivation). MON 89788 is shown to be substantially equivalent to traditional soybean, except for the introduced tolerance to glyphosate and, therefore, is unlikely to pose any threat to the EU environment or to require special measures for its containment. Furthermore, soybean volunteers can be easily controlled using currently available selective herbicides or by mechanical means. Therefore, no special measures are considered to be required in case of misuse or unintended release.

BNSDOCID: <XP_____2411357A__I_>

B. INFORMATION RELATING TO (A) THE RECIPIENT OR (B) (WHERE APPROPRIATE) PARENTAL PLANTS

1. Complete name

a) Family name Leguminosae
b) Genus *Glycine*
c) Species *max*
d) Subspecies Not applicable
e) Cultivar/breeding line A3244
f) Common name Soybean

2. a) Information concerning reproduction

(i) Mode(s) of reproduction

Soybean is a diploidized tetraploid (2n = 40) and is a self-pollinated species, propagated by seed.

The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals, one pistil and nine fused stamens with a single separate posterior stamen. The stamens form a ring at the base of the stigma and elongate one day before pollination, at which time the elevated anthers form a ring around the stigma. The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation and cross-pollination is usually less than one percent.

Pollination typically takes place on the day the flower opens. The pollen comes naturally in contact with the stigma during the process of anthesis. Anthesis normally occurs in late morning (usually between 10.00 and 11.00 am, depending on the environmental conditions). The pollen usually remains viable for 2-4 hours, after which it germinates. No viable pollen can be detected by late afternoon. Natural or artificial cross-pollination can only take place during the short time of the day that the pollen is viable.

A soybean plant can produce as many as 400 pods, with two to twenty pods at a single node. Each pod contains one to five seeds. Neither the seedpod, nor the seed, has morphological characteristics that would facilitate animal transportation.

(ii) Specific factors affecting reproduction

Soybean is a quantitative short day plant and hence flowers more quickly under short days. As a result, photoperiodism and temperature response are important in determining areas of cultivar adaptation.

During the reproductive stages of development, soybean plants are particularly sensitive to hydric and thermal (low temperature) stress which can cause significant flower abortion and yield loss. Soybeans do not yield well on acid soils and the addition of limestone may be required.

(iii) Generation time

Soybean is an annual crop which is planted in Europe in late spring (April to May). Pods develop in late summer (August) and harvesting is normally in September to October. The length of the cultural cycle is 100 to 160 days, depending on the variety and the region in which it is cultivated.

2 b) Sexual compatibility with other cultivated or wild plant species

Glycine is the only genus in the tribe Phaseoleae where species have diploid chromosome numbers of 40 and 80, but not 20. The unique chromosome number of *Glycine* is probably derived from diploid ancestors with base number 11, which have undergone aneuploid loss to base number 10. In the Family Leguminosae, only 10 of 71 genera are considered completely polyploid and *Glycine* is one of them. Soybean should be regarded as a stable tetraploid with diploidized genomes. Although soybean is a self-pollinated species, natural cross-pollination may occur.

Outcrossing with cultivated soybean species

Natural cross-pollination in soybean has been found to be generally very low. Cross-pollination frequencies vary with growing conditions, genotypes and location of the male parent in relation to the female parent. Insect activity does increase the outcrossing rate, but soybeans are generally not the preferred plant for pollinators.

Numerous studies on soybean cross-pollination have been conducted (with and without supplemental pollinators). Under natural conditions, cross-pollination among adjacent plants in a row or among plants in adjacent rows ranged from 0.03 to 3.62%. In experiments where supplemental pollinators (usually bees) were added to the experimental area, cross-pollination ranged from 0.5 to 7.74% in adjacent plants or adjacent rows. However, cross-pollination does not occur at these levels over long distances. Cross-pollination rates decrease to less than 1.5%

BNSDOCID: <XP_____2411357A__I_>

beyond one meter from the pollen source and decreases further with greater distances from the source. For example, the following outcrossing rates at extended distances have been reported: 0.05% at 5.4 m, 0% at 6.5 m and 0.02% at 8.2 m.

It has however to be noted that the scope of the current application does not include the cultivation of MON 89788 varieties in the EU. Therefore, any outcrossing between MON 89788 and cultivated *Glycine* varieties is highly unlikely.

Outcrossing with wild soybean species

From a taxonomic standpoint, both the wild annual species of subgenus *Soja* and the wild perennial species of subgenus *Glycine* are candidates for gene exchange with the cultivated soybean. No other genus is closely enough related to soybean to allow for the possibility of outcrossing. Therefore, the discussion below will concentrate on species of subgenus *Glycine* and *Soja*.

Hybridization with wild perennial species of subgenus Glycine

There are no wild relatives of subgenus *Glycine* in Europe. Therefore, the only opportunities for inter-subgeneric hybridization would occur in Australia, West, Central and South Pacific Islands, China, Russia, Japan, Indonesia, Korea, Papua New Guinea, Philippines and Taiwan, where those species are endemic. Nonetheless, there are no known reports of successful natural hybridization between cultivated soybean and these wild perennial species. All inter-subgeneric hybrids were obtained through *in vitro* seed culture. The F_1 hybrids were generally sterile and further progeny has been obtained only in a few cases and with great difficulty. Consequently, the possibility of gene transfer between cultivated soybean and wild species of subgenus *Glycine* does not exist.

Hybridization with the wild annual species of subgenus Soja

The wild annual species *G. soja*, found in China, Taiwan, Japan, Korea and Russia can hybridize naturally with the cultivated soybean, *G. max*, since they are both members of the subgenus *Soja*. Frequency of spontaneous cross-pollination in reciprocal combinations of *G. max* and *G. soja* varied from 0.73 (♀ *G. soja* × ♂ *G. max*) to 12.8% (♀ *G. max* × ♂ *G. soja*). Hybridization between female *G. soja* and male *G. max* was less successful than hybridization in the opposite direction. Species relationships in the subgenus *Soja* indicated that F_1 hybrids of *G. max* (2n = 40) and *G. soja* (2n = 40) carry similar genomes and are completely fertile or differ by a single reciprocal translocation. *G. gracilis*, known only from Northeast China, is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. *G. gracilis* may be an intermediate in the speciation of *G. max* from *G. soja* or a hybrid between *G. soja* and *G. max*. Interspecific fertile hybrids between *G. max* and *G. soja* and between *G. max* and *G. gracilis* have been easily obtained. It has however to be noted that the frequency of crop-to-wild introgression, which is defined as the permanent incorporation of genes from one

population/species to another after hybridization, is thought to be exceedingly low in soybean.

To conclude, gene transfer between cultivated soybean and wild species of subgenus *Soja* may occur, but not in Europe, where the wild relatives of subgenus *Soja* are not present.

3. Survivability

a) Ability to form structures for survival or dormancy

Soybean reproduces solely by means of seeds. Mature soybean seeds have no innate dormancy and are sensitive to cold. Due to the lack of dormancy (which is selected for in commercial soybean seed), soybean seeds germinate quickly with adequate temperature and moisture. As a consequence, all seed that might shatter and fall to the ground will eventually germinate and grow as a volunteer in the year following cultivation. However, volunteers are likely to be killed by frost during the autumn or early winter of the year they were produced. In case they should establish, volunteers do not compete well with the succeeding crop and can easily be controlled mechanically or chemically.

b) Specific factors affecting survivability

See Section B.3.a.

4. Dissemination

a) Ways and extent of dissemination

In theory, soybean dissemination may occur by means of seed dispersal or pollen dispersal.

However, during soybean harvesting, there are few seed lost due to the relatively large seed size. Furthermore, neither the soybean seedpod, nor the seed have morphological characteristics that would facilitate animal transportation.

Soybean pollen may also be considered as a vehicle for dissemination, but the pollen viability outside of the soybean flower is limited by the fact that soybean is a predominately self-pollinating plant with anthers enclosed within the keel of the corolla. However, natural cross-pollination may occur to a certain extent as discussed in B.2.a.

b) Specific factors affecting dissemination

See Section B.4.b.

BNSDOCID: <XP_____2411357A__I_>

5. **Geographical distribution and cultivation of the plant, including the distribution in Europe of the compatible species**

Soybean was domesticated in the eastern half of northern China around the 11th century B.C. or earlier and its cultivation subsequently extended throughout south-east Asia. Soybean cultivation was probably introduced in Europe starting in the late 16th and throughout the 17th century and in the United States of America (U.S.A.) in the 18th century. Today, soybean is grown as a commercial crop in over 35 countries. The major producers of soybeans are the U.S.A., Brazil, Argentina and China. In Europe, soybean is grown mainly in Italy and France. Outside of the EU, Romania, one of the accession countries, is also a large soybean producer.

Soybean is grown primarily for the production of seed, has a multitude of uses in the food and industrial sectors and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use.

There are no compatible species for cultivated soybean in Europe.

6. **In the case of plant species not normally grown in the Member State(s), description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts**

Not applicable, as soybean is grown in Europe.

7. **Other potential interactions, relevant to the GM plant, of the plant with organisms in the ecosystem where it is usually grown, or used elsewhere, including information on toxic effects on humans, animals and other organisms**

Soybean is known to interact with other organisms in the agricultural environment. Soybean is sensitive to a number of economically important diseases and insect predators and is susceptible to competition from surrounding weeds, which commonly compete with soybean for light, water and nutrients.

Soybean seed is known to contain a number of natural anti-nutritional components, which are completely or partially inactivated during processing. Trypsin (proteinase) inhibitors are known to have anti-nutritive properties in animals fed unprocessed soybeans. Other anti-nutrients include lectins, stachyose, raffinose and phytic acid. Some of these anti-nutrients relate to their impact on human nutrition, while others relate to animal nutrition in general including livestock.

Soybeans have been shown to contain endogenous proteins that elicit an allergenic response in humans. The prevalence of individuals with allergies to soybeans varies significantly between geographies. Allergenicity to soy proteins is generally a transient allergy of infancy or childhood, with allergenicity fairly rare in adults. However, allergenic reactions to soy proteins are mostly manifested in atopic symptoms and are rarely life-threatening.

C. INFORMATION RELATING TO THE GENETIC MODIFICATION

1. Description of the methods used for the genetic modification

Plasmid vector PV-GMGOX20 was used to produce MON 89788 by *Agrobacterium*-mediated tranformation. This transformation was conducted on the soybean cultivar A3244.

2. Nature and source of the vector used

The vector used to generate MON 89788 by *Agrobacterium*-mediated transformation is PV-GMGOX20. The genetic elements present in PV-GMGOX20 are described in Table 1.

3. Source of donor DNA, size and intended function of each constituent fragment of the region intended for insertion

The genetic elements of PV-GMGOX20 intended for insertion into the soybean genome comprised between the T-DNA borders are *FMV/Tsf1*, a chimeric promoter containing enhancer sequences to regulate constitutive expression; the *Tsf1* leader and intron sequence to enhance the expression of the *CTP2/cp4 epsps* coding sequence; the *CTP2* transit peptide to direct the transport of the CP4 EPSPS protein to the chloroplast; the *cp4 epsps* coding sequence encoding the CP4 EPSPS protein that confers tolerance to glyphosate and the *E9* 3' nontranslated sequence that directs transcriptional termination and polyadenylation of the *CTP2/cp4 epsps* mRNA.

A detailed description of each element can be found in Table 1.

BNSDOCID: <XP_____2411357A__I_>

Table 1. Summary of genetic elements in plasmid vector PV-GMGOX20

Genetic element	Position in plasmid	Function
		T-DNA
Intervening sequence	1-51	Sequences used in DNA cloning
P-*FMV/Tsf1*	52-1091	Chimeric promoter consisting of enhancer sequences from the *35S* promoter of the Figwort Mosaic Virus and the promoter from the *Tsf1* gene of *Arabidopsis thaliana* encoding the elongation factor EF-1 alpha
L-*Tsf1*	1092-1137	5' nontranslated leader (exon 1) from the *Tsf1* gene of *Arabidopsis thaliana* encoding the elongation factor EF-1 alpha
I-*Tsf1*	1138-1759	Intron from the *Tsf1* gene of *Arabidopsis thaliana* encoding the elongation factor EF-1 alpha
Intervening sequence	1760-1768	Sequences used in DNA cloning
TS-*CTP2*	1769-1996	Sequences encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS
CS-*cp4 epsps*	1997-3364	Codon optimized coding sequence of the *aroA* (*epsps*) gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein
Intervening sequence	3365-3406	Sequences used in DNA cloning
T-*E9*	3407-4049	3' nontranslated sequence from the ribulose-1,5-bisphosphate carboxylase small subunit (*RbcS2*) *E9* gene of pea (*Pisum sativum*)
Intervening sequence	4050-4092	Sequences used in DNA cloning
B-Left border	4093-4534	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
		Vector backbone
Intervening Sequence	4535-4620	Sequences used in DNA cloning
OR-*ori V*	4621-5017	Origin of replication from the broad host range plasmid RK2 for plasmid maintenance in *Agrobacterium*
Intervening Sequence	5018-6525	Sequences used in DNA cloning
CS-*rop*	6526-6717	Coding sequence for the repressor of primer protein for maintenance of plasmid copy number in *E. coli*
Intervening Sequence	6718-7134	Sequences used in DNA cloning
OR-*ori-PBR322*	7135-7763	Origin of replication from pBR322 for plasmid maintenance in *E. coli*
Intervening sequence	7764-8263	Sequences used in DNA cloning
aadA	8264-9152	Bacterial gene encoding an aminoglycoside-modifying enzyme, 3' (9)-O-nucleotidyl-transferase from the transposon Tn7
Intervening sequence	9153-9288	Sequences used in DNA cloning
		T-DNA
B-Right border	9289-9645	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
Intervening sequence	9646-9664	Sequences used in DNA cloning

D. INFORMATION RELATING TO THE GM PLANT

1. Description of the trait(s) and characteristics which have been introduced or modified

MON 89788 contains a fully functional intact gene encoding the CP4 EPSPS protein, which confers tolerance to glyphosate. Glyphosate has excellent weed control capabilities and well-known, favourable environmental and safety characteristics. However, the sensitivity of crop plants to glyphosate has prevented the in-season use of this herbicide over-the-top of crops. The extension of its use to allow in-season application in major crops such as soybean provides a novel weed control option for farmers.

2. Information on the sequences actually inserted or deleted

a) The copy number of all detectable inserts, both complete and partial

Molecular analysis was conducted to characterize the insert in MON 89788. Genomic DNA was analyzed using Southern blot to determine the insert number (number of insertions of the integrated DNA within the soybean genome), the copy number (the number of copies of the integrated DNA within one locus), the integrity and organization of the inserted promoters, coding sequences and 3' nontranslated sequence and the presence or absence of any other elements of plasmid vector PV-GMGOX20.

The molecular analysis shows that one intact copy of the *cp4 epsps* expression cassette is integrated at a single integration locus. No additional elements from the transformation vector PV-GMGOX20, linked or unlinked to the T-DNA, were detected in the genome of MON 89788. Additionally, no element of the PV-GMGOX20 backbone sequence was detected in MON 89788.

b) In case of deletion(s), size and function of the deleted region(s)

Not applicable.

c) Chromosomal location(s) of insert(s) (nucleus, chloroplasts, mitochondria, or maintained in a non-integrated form), and methods for its determination

The presence of the MON 89788 insert in the soybean nuclear genome is best shown by the Chi square (χ^2) analysis of the segregation data. The results of the χ^2 analysis indicate that a single insert is integrated in the plant nuclear genome and is stably inherited as a single locus, following a Mendelian one-locus model.

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d) The organisation of the inserted genetic material at the insertion site

The MON 89788 insert consists of the *cp4 epsps* gene cassette containing the *FMV/Tsf1* promoter, the *Tsf1* leader and *Tsf1* intron sequences, the *CTP2* chloroplast transit peptide sequence, the *cp4 epsps* coding sequence and the *E9* 3' nontranslated sequence.

3. Information on the expression of the insert

a) Information on developmental expression of the insert during the life cycle of the plant

MON 89788 produces one functional protein, CP4 EPSPS, providing tolerance to glyphosate. CP4 EPSPS protein levels were determined in over-season leaf (OSL), seed, root and forage tissues derived from MON 89788 plants produced in replicated field trials across five Argentinean and five U.S.A. locations during the 2004-2005 and 2005 growing seasons, respectively.

For MON 89788, the mean CP4 EPSPS protein levels across Argentinean sites for OSL1, OSL2, OSL3, OSL4, seed, root and forage were 280, 340, 310, 460, 170, 100 and 290 µg/g dw, respectively. The mean CP4 EPSPS protein levels across U.S.A. sites for OSL1, OSL2, OSL3, OSL4, seed, root and forage were 300, 340, 330, 290, 150, 74 and 220 µg/g dw, respectively. It is therefore concluded that the CP4 EPSPS protein expression levels measured in each tissue are comparable across both growing seasons and geographies.

b) Parts of the plant where the insert is expressed

The expression of the CP4 EPSPS protein occurs throughout the plant since the *FMV/Tsf1* promoter has been shown to drive constitutive expression of the encoded protein in genetically modified plants.

As seed and forage are the most relevant tissues for the safety assessment of MON 89788, protein levels in these tissues were estimated in field trials conducted in U.S.A. and Argentina. However, protein levels in overseason leaves and roots were also determined, as described in 3(a).

4. Information on how the GM plant differs from the recipient plant in

a) Reproduction

Based on centuries of experience with traditional, domesticated soybean in the EU, there is no potential for soybean to be invasive of natural habitats or persist in the agronomic environment without the aid of human intervention. Soybean is known as a poor competitor, which outside of cultivation has no meaningful impact on the environment.

Agronomic data collected from trials conducted with MON 89788 in 2005 at 17 sites in the U.S.A. in the major soybean producing geographies have demonstrated that MON 89788 has not been altered in survival,

multiplication and dissemination characteristics when compared to traditional soybean.

On the basis of the studies described above, it is possible to conclude that no differences in the mode or rate of reproduction, dissemination, survivability or other agronomic, phenotypic or ecological characteristics are expected in MON 89788 and that MON 89788 is equivalent to traditional soybean in its phenotypic and agronomic behaviour, except for the glyphosate-tolerance trait.

b) Dissemination

See Section D.4.a.

c) Survivability

See Section D.4.a.

d) Other differences

See Section D.4.a.

5. Genetic stability of the insert and phenotypic stability of the GM plant

The results of the χ^2 analysis show that a single insert is integrated in the plant nuclear genome of MON 89788 and is stably inherited, following a Mendelian one-locus model.

Additionally, on a phenotypic level, since the initial development and breeding of MON 89788, the glyphosate-tolerance trait has been consistently inherited in a Mendelian fashion for several generations without reported instability.

The genetic stability of MON 89788 has been further confirmed by Southern blot analyses.

6. Any change to the ability of the GM plant to transfer genetic material to other organisms

a) Plant to bacteria gene transfer

No elements known to be involved in DNA mobility have been included in the inserted DNA. Therefore, in comparison to traditional soybean, no changes are to be expected in the ability of the GM plant to exchange genetic material with bacteria.

b) Plant to plant gene transfer

Based on the observation that reproductive morphology in MON 89788 is unchanged compared to traditional soybean and that pollen production and pollen viability were unaffected by the genetic modification, the out-crossing frequency to other soybean varieties or to wild relatives (which are not present in the EU) would be unlikely to be different for MON 89788, when compared to traditional soybean varieties.

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However, the scope of the current application does not include the cultivation of MON 89788 varieties in the EU.

7. Information on any toxic, allergenic or other harmful effects on human or animal health arising from the GM food/feed

7.1 Comparative assessment

Choice of the comparator

MON 89788 was compared to A3244, a traditional soybean variety with background genetics similar to MON 89788.

7.2 Production of material for comparative assessment

a) number of locations, growing seasons, geographical spread and replicates

Compositional analyses were conducted on MON 89788 soybean seed and forage grown under replicated field conditions at five U.S.A. and five Argentinean locations during the 2005 and 2004-2005 growing seasons, respectively. Field trials were located in regions that are representative of commercial soybean production. MON 89788 was compared to A3244, a traditional soybean variety with background genetics similar to MON 89788 and to traditional soybean varieties. MON 89788 was found to be compositionally equivalent to traditional soybeans and thus as safe as traditional soybeans for uses in food and feed applications.

b) the baseline used for consideration of natural variations

In both locations, 12 traditional soybean reference varieties grown in the same plots were included as references to provide data for the development of a 99% tolerance interval for each analyte analyzed. This interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of traditional soybean varieties.

7.3 Selection of material and compounds for analysis

The key nutrients and other nutritionally important components that were selected for analysis in the compositional studies were chosen on the basis of internationally accepted guidance provided by the OECD on compositional considerations for new varieties of soybean.

7.4 Agronomic traits

Field trials with MON 89788 were conducted and the set of agronomic observations supports the conclusion that from an agronomic and phenotypic (morphological) point of view, MON 89788 is equivalent to traditional soybean, except for the introduced glyphosate tolerance trait (*see* Section D.4.).

7.5 Product specification

MON 89788 contains a functionally intact gene cassette encoding the CP4 EPSPS protein, which confers tolerance to glyphosate. MON 89788 will be imported into the EU in mixed shipments of soybean products, produced in other world areas, for use by operators that have traditionally been involved in the commerce, processing and use of soybean and soybean derived products in the EU.

The presence of the glyphosate-tolerance trait in soybeans or soybean derived products can be identified by employing different techniques. Southern blot or PCR techniques can identify the inserted nucleotide sequences, while ELISAs have been developed to detect the presence of the CP4 EPSPS protein in individual MON 89788 plants or in specific tissues. A MON 89788-specific PCR assay allowing the identification and the quantification of MON 89788 has been developed and provided to the Joint Research Centre (JRC), acting as the Community Reference Laboratory (CRL).

7.6 Effect of processing

As MON 89788 is compositionally equivalent to traditional soybean, the use of MON 89788 for the production of foods and feeds is not expected to be different from that of traditional soybean. The production and processing of MON 89788 does not differ from the production and processing of the equivalent foods and feeds, originating from traditional soybean.

7.7 Anticipated intake/extent of use

There are no anticipated changes in the intake and/or extent of use of soybean or derived products for use as or in food or feed as a result of the addition of MON 89788 to the soybean supply. MON 89788 is expected to replace a portion of current soybean such that its intake or use will represent some fraction of the total products derived from soybean.

7.8 Toxicology

7.8.1 Safety assessment of newly expressed proteins

MON 89788 contains the *cp4 epsps* expression cassette that produces the CP4 EPSPS protein. The assessment of human and animal safety of the CP4 EPSPS protein includes (i) the safety of the donor organism, *Agrobacterium* sp. strain CP4; (ii) the similarity of CP4 EPSPS to other EPSPSs naturally present in foods with a long history of safe consumption; (iii) the presence of CP4 EPSPS in commercial food and feed crops; (iv) the bioinformatic comparisons of the CP4 EPSPS protein to known toxic or pharmacologically active proteins and (v) an acute oral toxicity study with CP4 EPSPS in mice.

Agrobacterium sp. strain CP4 was chosen as the donor organism because this bacterium exhibited tolerance to glyphosate by producing a naturally glyphosate-tolerant EPSPS protein (CP4 EPSPS). *Agrobacterium* species are not known for human or animal pathogenicity and are not commonly allergenic.

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The CP4 EPSPS protein is a member of the EPSPS family, a well-known class of proteins that are ubiquitous in nature, as they are present in algae, plants, fungi and bacteria, but not in animals. The similarity of the CP4 EPSPS protein to EPSPSs in a variety of foods supports extensive human consumption of the family of EPSPS proteins and the lack of health concerns.

CP4 EPSPS-containing crops have been commercialized and consumed as foods and feeds since their initial introduction in 1996. Roundup Ready soybean 40-3-2, which produces the same CP4 EPSPS protein contained in MON 89788, represents a significant portion of commodity crops containing CP4 EPSPS protein. Globally, Roundup Ready soybean 40-3-2 was produced on approximately 54.4 million hectares in 2005, which represented 60% of global soybean area. Consequently, 60% of the soybean and soybean products consumed globally are likely to contain the CP4 EPSPS protein. In the EU, Roundup Ready soybean 40-3-2 has been approved for import and use as from 1996. Since its authorization Roundup Ready soybean 40-3-2 and derived products represent a significant portion of the soybean products used in Europe. In addition to soybean, the CP4 EPSPS protein is also expressed in other Roundup Ready crops that are used primarily as food and feed sources, such as Roundup Ready maize NK603, Roundup Ready oilseed rape and Roundup Ready cotton. The cumulative experience with release of crops expressing the CP4 EPSPS protein showed no evidence of any harmful or undesirable effects associated with Roundup Ready crops or with the CP4 EPSPS protein. This demonstrates the history of safe use of Roundup Ready crops and of the CP4 EPSPS protein they produce, including the safe use of MON 89788.

Finally, the CP4 EPSPS protein has been shown not to be homologous to known toxins or pharmacologically-active proteins and no indications of toxicity were reported in mice administered the CP4 EPSPS protein by oral gavage.

On the basis of the information presented, it is therefore possible to conclude that the CP4 EPSPS protein is safe and poses no concerns for humans, animals and the environment.

7.8.2 Testing of new constituents other than proteins

Soybean has a long history of safe use and consumption around the world. MON 89788 has been shown to be compositionally equivalent to traditional soybean. Therefore, no testing of any constituent other than the introduced protein is indicated.

7.8.3 Information on natural food and feed constituents

Soybean is known to contain a number of natural anti-nutritional components, such as trypsin inhibitors, lectins, isoflavones (daidzein, genistein and glycitein), stacchyose, raffinose and phytic acid, which are inactivated when the beans are toasted or heated during processing. Nonetheless, these antinutrients were evaluated

in MON 89788 compositional analyses and their levels were demonstrated to be comparable in MON 89788 and in traditional soybean.

7.8.4 Testing of the whole GM food/feed

Compositional analyses and comparative phenotypic assessments have demonstrated that MON 89788 is substantially equivalent to traditional soybean, with the exception of the introduced glyphosate-tolerance trait, which is conferred by the production of the CP4 EPSPS protein.

The CP4 EPSPS protein produced in MON 89788 is shown to be safe for consumption by humans and animals. The safety of MON 89788 has been further confirmed by a 90-day feeding study in rats and by a 42-day feeding study in broiler chickens.

7.9 Allergenicity

7.9.1 Assessment of allergenicity of the newly expressed protein

It is unlikely that the CP4 EPSPS protein will cause allergenic concerns due to the following considerations: (1) the CP4 EPSPS protein was obtained from *Agrobacterium* species and therefore, it does not originate from a source known to be allergenic; (2) the CP4 EPSPS protein represents approximately 0.04% of the total protein in MON 89788 seed. Since this constitutes only a small portion of the total protein in MON 89788 seed, CP4 EPSPS is not likely to be an allergenic protein; (3) the updated bioinformatic analysis confirmed that the CP4 EPSPS protein shows no structurally significant amino acid sequence similarity to any known protein allergens, gliadins or glutenins. Therefore, it is unlikely that CP4 EPSPS contains allergenic epitopes; (4) the CP4 EPSPS protein is extremely labile to digestion in an *in vitro* pepsin digestion assay, a characteristic shared among proteins with a history of safe consumption. Thus, using the best methodology available today, it can be concluded that the allergenic potential of the CP4 EPSPS protein is negligible and therefore, this protein does not pose a significant allergenic risk.

7.9.2 Assessment of allergenicity of the whole GM plant or crop

Soybean is known to cause food allergies on certain individuals. Therefore, to assess whether MON 89788 has altered endogenous allergenic potential compared to traditional soybean, a study was conducted to determine binding levels of human IgE antibody to protein extracts prepared from MON 89788 and A3244, a traditional soybean with background genetics similar to MON 89788. In addition, 24 commercially available soybean varieties were included in the study for the establishment of the tolerance interval for each serum and they represent the normal variations of responses expected from soybeans.

The results of this study indicate that MON 89788 has similar IgE-binding values to A3244 and in all cases, MON 89788 IgE-binding

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values are within the ranges established by commercially available soybean varieties. Thus, it is concluded that MON 89788 soybean has no greater allergenic potential than soybean varieties that are currently on the market.

7.10 Nutritional assessment of GM food/feed

7.10.1 Nutritional assessment of GM food

MON 89788 was shown to be compositionally equivalent to traditional soybean. The introduced glyphosate-tolerance trait is of agronomic interest and is not intended to change any nutritional aspects of this soybean. Therefore, anticipated dietary intake of soybean-derived foods is not expected to be altered in the EU and no nutritional imbalances are expected as a result.

7.10.2 Nutritional assessment of GM feed

MON 89788 was demonstrated to be compositionally equivalent to traditional soybean. The safety assessment of MON 89788 showed that this glyphosate-tolerant soybean does not pose any adverse effects for humans and animals. The nutritional value of MON 89788 was assessed by a 42-day feeding broiler chicken feeding study, which established the nutritional equivalence of this soybean to traditional soybean for use as feed. This study further confirmed the absence of any pleiotropic or unanticipated effects resulting from the introduction of the glyphosate-tolerance trait into the soybean genome.

7.11 Post-market monitoring of GM food/feed

There are no intrinsic hazards related to MON 89788 as no signs of adverse or unanticipated effects have been observed in a number of safety studies, including animal feeding studies using doses of administration that are orders of magnitude above expected consumption levels. The pre-market risk characterization for food and feed use of MON 89788 demonstrates that the risks of consumption of MON 89788 or its derived products are consistently negligible and no different from the risks associated with the consumption of traditional soybean. As a consequence, specific risk management measures are not indicated, and post-market monitoring of the use of soybean for food and feed is not considered appropriate.

8. Mechanism of interaction between the GM plant and target organisms (if applicable)

Not applicable. MON 89788 is tolerant to glyphosate and, as such, does not have any target organisms.

9. Potential changes in the interactions of the GM plant with the biotic environment resulting from the genetic modification

9.1 Persistence and invasiveness

Based on centuries of experience with traditional, domesticated soybean in Europe, there is no potential for soybean to be invasive of natural habitats or persist in the environment without the aid of human intervention.

MON 89788 is substantially equivalent to traditional soybean, except for the introduced glyphosate-tolerance trait. Field trial data demonstrated that this soybean has not been altered in its phenotypic, agronomic, reproductive, survival and dissemination characteristics when compared to traditional soybean. In the unlikely event of establishment of a MON 89788 plant, *e.g.* from a seed spilt in the environment, its introduced trait would have negligible consequences for the environment.

Therefore, the likelihood of unintended spreading of MON 89788 into the environment is negligible.

9.2 Selective advantage or disadvantage

It was previously demonstrated that the introduced genetic sequences in MON 89788 did not lead to any biologically meaningful alterations of other phenotypic characteristics, such as plant growth and development, morphology, agronomic performance, composition, nutritional value or safety characteristics, when compared to traditional soybean. It was concluded that MON 89788 is substantially equivalent to traditional soybean, with the exception of the intentionally introduced glyphosate-tolerance trait. Therefore, the assessment of any conferred competitive advantages is limited to the glyphosate-tolerance trait, as no other new traits were introduced in this soybean.

The only selective advantage of MON 89788 that results from the genetic modification is the plant's tolerance to glyphosate. This 'selective advantage' over other plants, however, is the basis of the Roundup Ready agronomic system and only applies where the crop is treated with a herbicide containing the active ingredient glyphosate, which are predictable conditions, that are spatially limited, short in duration, and with negligible consequences to natural environments. In environments where the selective pressure from glyphosate applications is absent, the glyphosate-tolerance trait does not confer a selective advantage or disadvantage and would not be of direct competitive importance to wild plants, nor indirectly for wildlife interacting with those wild plants. Even when spillage of MON 89788 seed would result in the short survival of some soybean volunteers, it would not represent a meaningful advantage over wild plants since MON 89788, like any other soybean, is a poor competitor in European conditions and the likelihood for spilt soybeans to survive and establish is negligible.

Therefore, the likelihood is negligible for the introduced trait in MON 89788 to confer any meaningful competitive advantage or disadvantage of relevance to the environment.

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9.3 Potential for gene transfer

There is no potential for gene transfer from MON 89788 to wild plant species in the EU and negligible likelihood for gene transfer to other soybean crops, as this application is not for consent to cultivate MON 89788 varieties in the EU.

Moreover, as the likelihood of accidentally spilt MON 89788 seed to germinate, establish, mature and flower is very low, as soybeans are predominantly self-pollinated and the majority of the remaining soybean pollen are largely confined to short distances from the source plant, the transfer of the introduced trait to neighbouring soybean plants through cross-pollination is negligible. Furthermore, in the highly unlikely case where a trait would be transferred, the risk of the trait to be the cause of any meaningful competitive advantage or disadvantage that could impact the receiving environment is negligible.

In light of the above, the likelihood of out-crossing of the introduced trait is negligible in the EU.

9.4 Interactions between the GM plant and target organisms

No characteristics could be identified which may cause an adverse environmental effect. MON 89788 is tolerant to glyphosate and, as such, has no target organisms with which to interact, either directly or indirectly.

9.5 Interactions of the GM plant with non-target organisms

Given the scope of the current application, which does not include the cultivation of MON 89788 varieties in the EU, the likelihood for direct or indirect interactions of this soybean with non-target organisms is considered to be negligible. In addition, even if incidental spillage of MON 89788 seed during import, storage, transport or use would lead to the short survival of MON 89788 plants, the newly produced protein in MON 89788, CP4 EPSPS, is not novel in the environment. The *cp4 epsps* coding sequence introduced into MON 89788 was derived from *Agrobacterium* sp. strain CP4, a common bacterium in the soil. Endogenous plant EPSPSs and the introduced CP4 EPSPS protein belong to a larger family of EPSPS proteins, which form a class of enzymes that are ubiquitous in all compartments of nature as they are found in bacteria, fungi, algae and all higher plants. Any non-target organisms in the environment that could be exposed to CP4 EPSPS from incidentally spilt MON 89788 seed or from any resulting volunteers interacting with the crop have co-evolved in close interaction with a wide spectrum of green plants and microorganisms and therefore have historically been exposed to members of this safe class of proteins. Based on the ubiquitous occurrence of natural EPSPSs and the history of safe use of CP4 EPSPS-producing crops such as Roundup Ready soybean 40-3-2, Roundup Ready oilseed rape and Roundup Ready cotton, which produce the same CP4 EPSPS protein present in MON 89788, it is highly unlikely that the CP4 EPSPS or any other EPSPS protein would possess biological activity towards non-target organisms such as plants, microorganisms, invertebrates or vertebrates.

To conclude, there is negligible risk for harmful effects of MON 89788 on

non-target organisms, either through direct or indirect interactions of this soybean or through contact with the newly expressed protein.

9.6 Effects on human health

The likelihood for any adverse effects occurring in humans as a result of their contact with MON 89788 is no different from traditional soybean. MON 89788 is substantially equivalent to traditional soybean, except for the introduced glyposate-tolerance trait, which is imparted by the production of the CP4 EPSPS protein. The safety of the CP4 EPSPS protein has been extensively investigated. The CP4 EPSPS protein present in MON 89788 is similar to EPSPS proteins, a class of enzymes that is ubiquitous in all compartments of nature and benefit from a long history of safe use, and is identical to the CP4 EPSPS protein produced in Roundup Ready soybean 40-3-2 and Roundup Ready oilseed rape, which have been commercialized and safely used for human and animal consumption since 1996 in several world areas, including Europe. The safety of the CP4 EPSPS protein to humans is further confirmed by (1) lack of acute toxicity as determined in a mouse acute gavage study, (2) rapid digestion in simulated gastric fluids, (3) lack of homology with known protein toxins and (4) lack of homology with known allergens. The safety of MON 89788 is corroborated by animal feeding studies in the rat and in broiler chickens using MON 89788 containing diets, demonstrating the absence of any toxic or pleiotropic effects linked to the genetic modification.

Therefore, the risk of any change in the occupational health aspects of this soybean is negligible.

9.7 Effects on animal health

Based on centuries of experience with traditional, domesticated soybean in Europe, there is a very low potential for soybean to cause any adverse health effects in livestock animals.

MON 89788 is substantially equivalent to traditional soybean, except for the introduced glyphosate-tolerance trait imparted by the CP4 EPSPS protein. As previously discussed, the CP4 EPSPS protein belongs to the ubiquitous class of EPSPS enzymes. Based on the presence of EPSPS enzymes in algae, plants, fungi and bacteria, it is unlikely that these proteins will possess any harmful properties or biological activity towards livestock animals.

In addition, the CP4 EPSPS protein has been shown to have no significant homology to known allergens or protein toxins and its non-hazardous nature has been assessed by extensive safety studies, including an acute gavage study in mice. Furthermore, the CP4 EPSPS protein produced in MON 89788 is identical to the full length, mature CP4 EPSPS protein found in Roundup Ready soybean 40-3-2 and other glyphosate-tolerant crops with established history of safe human and animal consumption. In Europe, experience with domestic livestock fed on Roundup Ready soybean 40-3-2 meal from the millions of hectares grown in the U.S.A. and other producing countries and imported into the EU since 1996, has not provided any indication of potential adverse

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effects in livestock.

Finally, the safety and wholesomeness of MON 89788 was established by feeding studies in rats and broiler chickens using MON 89788-containing diets.

Therefore, it is possible to conclude that MON 89788 is as safe and as wholesome as traditional soybean and the risk posed by this soybean to the feed/food chain is negligible.

9.8 Effects on biogeochemical processes

As for traditional soybean, during transport or storage MON 89788 seed could spill and fall to the ground. Although such seed could eventually germinate if the local soil and environmental conditions are favourable, this soybean is a poor competitor and does not persist as a weed. Environmental conditions at the sites of handling are, however, unlikely to be conducive to germination, growth and reproduction of soybeans that is incidentally released.

There is no evidence that MON 89788 plants would be any different from traditional soybean regarding their direct influence on biogeochemical processes or nutrient levels in the soil, as MON 89788 has equivalent growth and development, morphology, plant health and survival characteristics to traditional soybean. Furthermore, any indirect interactions of the GMO with other organisms in the vicinity of an incidental release of the grain are not likely to cause hazardous effects on the biogeochemical processes in the soil. As previously discussed, CP4 EPSPS is widely present in the environment.

In conclusion, as for traditional soybean, it is highly unlikely that there would be any significant immediate or delayed adverse effects from MON 89788 on the biogeochemical processes in the soil.

9.9 Impacts of the specific cultivation, management and harvesting techniques

Not applicable. This application is for consent to import MON 89788 grain in the EU and for the use of this soybean as any other soybean, excluding the cultivation of MON 89788 varieties in the EU.

10. Potential interactions with the abiotic environment

MON 89788 was shown to be substantially equivalent to traditional soybean, except for the introduced glyphosate-tolerance trait, imparted by the production of the CP4 EPSPS protein. Although CP4 EPSPS is a newly introduced protein in soybean, it has a history of safe use and has no known negative interactions with the abiotic environment. The CP4 EPSPS protein in MON 89788 is innocuous and belongs to a large class of EPSPS proteins that are ubiquitous in nature and benefit from a long history of safe use. The safety of the CP4 EPSPS protein is further confirmed by the history of safe use of CP4 EPSPS crops.

No deleterious impact of MON 89788 on the abiotic environment is expected to result from the import, processing or use of this product for food and feed in the EU.

11. Environmental monitoring plan (not if application concerns only food and feed produced from GM plants, or containing ingredients produced from GM plants and if the applicant has clearly shown that environmental exposure is absent or will be at levels or in a form that does not present a risk to other living organisms or the abiotic environment)

11.1 General (risk assessment, background information)

As required by Article 5(5)(b) and 17(5)(b) of Regulation (EC) No 1829/2003, a environmental monitoring plan in accordance to Annex VII of Directive 2001/18/EC is included.

11.2 Interplay between environmental risk assessment and monitoring

An environmental risk assessment (e.r.a.) of MON 89788 was undertaken as required by Articles 5(5) and 17(5) of Regulation (EC) No 1829/2003. Analysis of the characteristics of MON 89788 has shown that the risk for potential adverse effects on human health and the receiving environment, resulting from the proposed use of MON 89788 in the EU is consistently negligible. Therefore, the overall environmental risk posed by this genetically modified higher plant is negligible, and no specific strategies for risk management and no case-specific post-market monitoring actions are considered required.

11.3 Case-specific GM plant monitoring (approach, strategy, method and analysis)

As the overall environmental risk posed by this genetically modified higher plant is negligible, and as the conclusions of the environmental risk assessment are derived from the results of scientific studies, rather than major assumptions, no case-specific post-market monitoring actions, typically aimed at testing assumptions made in this assessment, would be warranted or required.

11.4 General surveillance of the impact of the GM plant (approach, strategy, method and analysis)

Any potential adverse effects of MON 89788 on human health and the environment, which were not anticipated in the e.r.a., can be addressed under the general surveillance. General surveillance is largely based on routine observation and implies the collection, scientific evaluation and reporting of reliable scientific evidence, in order to be able to identify whether unanticipated, direct or indirect, immediate or delayed adverse effects have been caused by the placing on the market of a genetically modified (GM) crop in its receiving environment.

In order to allow detection of the broadest possible scope of unanticipated adverse effects, general surveillance is performed by either selected, existing networks, or by specific company stewardship programmes, or by a combination of both. The consent holder will ensure that appropriate technical information on MON 89788 and relevant legislation will be available for the relevant networks, in addition to further relevant information from a number of sources, including industry and government websites, official registers and government publications.

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Following the approval of this soybean in the EU, the consent holder will approach key stakeholders and key networks of stakeholders of the product (including international grain traders, soybean processors and users of soybean seed for animal feed) and inform them that the product has been authorized. The consent holder will request key stakeholders and networks for their participation in the general surveillance of the placing on the market of this soybean, in accordance with the provisions of Directive 2001/18/EC and the consent. Key stakeholders and networks will be requested to be aware of their use of this soybean and to inform the consent holder in case of potential occurrence of any unanticipated adverse effects to health or the environment, which they might attribute to the import or use of this product. Appropriate technical information on MON 89788 will be provided to them.

Where there is scientifically valid evidence of a potential adverse effect (whether direct or indirect), linked to the genetic modification, then further evaluation of the consequence of that effect should be science-based and compared with available baseline information. Relevant baseline information will reflect prevalent use practices and the associated impact of these practices on the environment. Where scientific evaluation of the observation confirms the possibility of an unanticipated adverse effect, this would be investigated further to establish a correlation, if present, between the use of MON 89788 and the observed effect. The evaluation should consider the consequence of the observed effect and remedial action, if necessary, should be proportionate to the significance of the observed effect.

11.5 Reporting the results of monitoring

Monsanto will submit an annual General Surveillance Report containing information obtained from participating networks, and/or in case of an effect that was confirmed. If information that confirms an adverse effect which alters the existing risk assessment becomes available, Monsanto will submit a Report, consisting of a scientific evaluation of the potential adverse effect and a conclusion on the safety of the product. The report will also include, where appropriate, the measures that were taken to ensure the safety of human or livestock health and/or the environment.

12. Detection and event-specific identification techniques for the GM plant

Southern blot or PCR techniques can be employed for the detection and identification of the inserted nucleotide sequences. ELISAs have been developed and can be used to detect the CP4 EPSPS protein in individual plants. A MON 89788-specific PCR-based assay allowing the identification and quantification of MON 89788 has been provided to the Joint Research Centre (JRC), acting as the Community Reference Laboratory (CRL).

E. INFORMATION RELATING TO PREVIOUS RELEASES OF THE GM PLANT AND/OR DERIVED PRODUCTS

1. **History of previous releases of the GM plant notified under Part B of the Directive 2001/18/EC and under Part B of Directive 90/220/EEC by the same notifier**

a) Notification number There is no history of release of MON 89788 in the EU.
b) Conclusions of post-release monitoring Not applicable.
c) Results of the release in respect to any risk to human health and the environment (submitted to the Competent Authority according to Article 10 of Directive 2001/18/EC) Not applicable.

2. **History of previous releases of the GM plant carried out outside the Community by the same notifier**

a) Release country MON 89788 has been field tested in the U.S.A. since 2001, in Argentina in the 2004-2005 growing season, in Chile in the 2005-2006 growing season and in Japan in 2006.
b) Authority overseeing the release U.S.A.: United States Department of Agriculture (USDA). Argentina: Secretary of Agriculture (SAGPyA) – CONABIA. Japan: Ministry of Agriculture Fisheries and Forestry. Chile: Agricultural and Livestock Service (SAG).
c) Release site U.S.A.: in the major soybean growing states and Puerto Rico. Argentina: Buenos Aires, Cordoba, Santa Fe. Japan: Ibaraki Prefecture. Chile: Graneros.
d) Aim of the release U.S.A./Argentina/Chile: efficacy, yield, breeding. Japan: stage III environmental assessment.
e) Duration of the release U.S.A./Argentina/Chile/Japan: 12 months.

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f) Aim of post-releases monitoring U.S.A./Argentina: assess for volunteers.
g) Duration of post-releases monitoring U.S.A./Argentina: 12 months.
h) Conclusions of post-release monitoring U.S.A./Argentina: in general, no volunteers have been observed since soybean is an annual crop. In southern geographies such as Puerto Rico, volunteers were eliminated to prevent occurrence in subsequent crops.
i) Results of the release in respect to any risk to human health and the environment Field-testing provided no evidence that MON 89788 or derived products would be the cause of any adverse effects to human health or to the environment.

3. **Links (some of these links may be accessible only to the competent authorities of the Member States, to the Commission and to EFSA):**

a) Status/process of approval The JRC websites http://gmoinfo.jrc.it/gmc_browse.asp and http://gmo-crl.jrc.it/statusofdoss.htm and the EFSA website http://www.efsa.eu.int/science/gmo/gm_ff_applications/catindex_en.html provide publicly accessible links to up-to-date databases on the regulatory progress of notifications under Directive 2001/18/EC and applications under Regulation (EC) No 1829/2003, including the Monsanto dossier for MON 89788.
b) Assessment Report of the Competent Authority (Directive 2001/18/EC) A notification for MON 89788 according to Directive 2001/18/EC has not been submitted by Monsanto Company.
c) EFSA opinion No EFSA opinion is available at the time of submission of this application.
d) Commission Register (Commission Decision 2004/204/EC) http://europa.eu.int/comm/food/dyna/gm_register/index_en.cfm
e) Molecular Register of the Community Reference Laboratory/Joint Research Centre Information on detection protocols is posted at http://gmo-crl.jrc.it/

f) Biosafety Clearing-House (Council Decision 2002/628/EC)

The publicly accessible portal site of the Biosafety Clearing-House (BCH) can be found at http://bch.biodiv.org/

g) Summary Notification Information Format (SNIF) (Council Decision 2002/812/EC)

EFSA provides a link to the publicly accessible summary of this application under Regulation (EC) No 1829/2003 at http://www.efsa.eu.int/science/gmo/gm_ff_applications/catindex_en.html.

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(71) Applicant *(for all designated States except US)*: **MONSANTO TECHNOLOGY LLC** [US/US]; 800 N. Lindbergh Boulevard, St. Louis, MO 63167 (US).

(72) Inventors; and

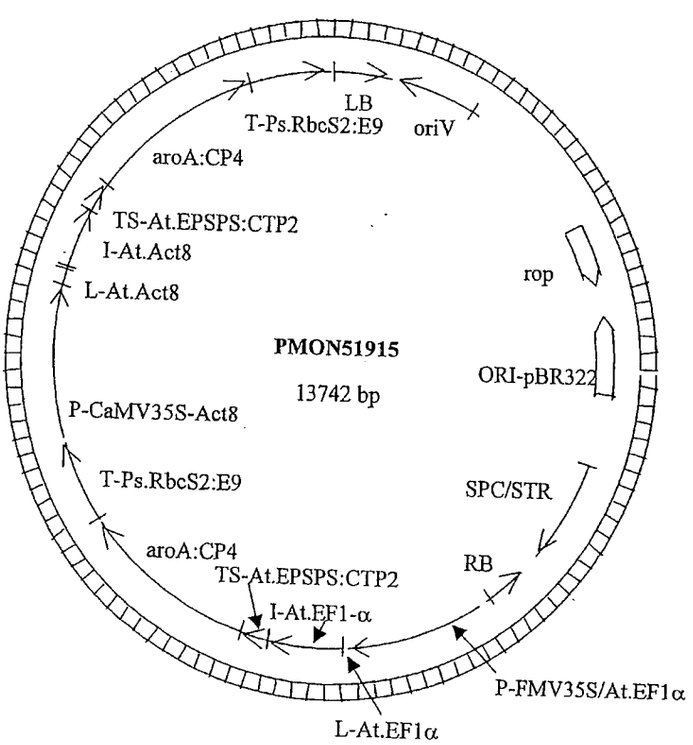
(75) Inventors/Applicants *(for US only)*: **CERNY, R., Eric** [US/US]; 1141 Richland Drive, Cheterfield, MO 63017 (US). **DUONG, Can** [US/US]; 2030 Brown Road, St. Louis, MO 63114 (US). **HART, Jesse, L.** [US/US]; 7382 Pershing Avenue #2W, St. Louis, MO 63130 (US). **HUBER, Scott, A.** [US/US]; 681 Hidden Path Apt. D, Creve Coeur, MO 63141 (US). **KRIEB, Rachel, L.** [US/US]; 282 S. 9th Street, Wood River, IL 62095 (US). **LISTELLO, Jennifer, J.** [US/US]; 6 Cherrywood Parc Court, O'Fallon, MO 63366 (US). **MARTENS, Amy, B.** [US/US]; 9225 Kit Drive, St. Louis, MO 63123 (US). **SAMMONS, Bernard** [US/US]; 380 Lafayette Court, Ellisville, MO 63011 (US).

(74) Agent: **KAMMERER, Patricia, A.**; Howrey Simon Arnold & White, LLP, 750 Bering Drive, Houston, TX 77057-2198 (US).

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[Continued on next page]

(54) Title: COTTON EVENT MON 88913 AND COMPOSITIONS AND METHODS FOR DETECTION THEREOF



(57) Abstract: The present invention provides a cotton plant event MON 88913 compositions and seed. Also provided are assays for detecting the presence of the cotton plant event MON 88913 based on a DNA sequence and the use of this DNA sequence as a molecular marker in a DNA detection method.

WO 2004/072235 A2

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COTTON EVENT MON 88913 AND COMPOSITIONS AND METHODS FOR DETECTION THEREOF

[0001] This application claims benefit of U.S. Provisional Application No. 60/447,184, filed February 12, 2003, the entire contents of which are incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of plant molecular biology. More specifically, the invention relates to a glyphosate tolerant cotton event MON 88913 and to assays and methods for detecting the presence of cotton event MON 88913 DNA in a plant sample and compositions thereof.

BACKGROUND OF THE INVENTION

[0003] Cotton is an important fiber crop in many areas of the world. The methods of biotechnology have been applied to cotton for improvement of the agronomic traits and the quality of the product. The method of introducing transgenes into cotton plants has been demonstrated in U.S. Patent No. 5,004,863. One such agronomic trait important in cotton production is herbicide tolerance, in particular, tolerance to glyphosate herbicide. This trait has been introduced into cotton plants and is a successful product now used in cotton production. The current commercial Roundup Ready® cotton event (1445) provides excellent tolerance to glyphosate, the active ingredient in Roundup®, through the four-leaf stage (Nida *et al.*, J. Agric. Food Chem. 44:1960-1966, 1996; Nida *et al.*, J. Agric. Food Chem. 44:1967-1974, 1996). However, foliar application beyond the four-leaf stage must be limited due to insufficient tolerance in male reproductive tissues in certain environmental conditions. This lack of male reproductive tolerance appears to be a result of insufficient CP4 EPSPS expression in critical tissues, higher sensitivity of these tissues to glyphosate, and accumulation of high amounts of glyphosate in these strong sink tissues (Pline *et al.*, Weed Sci. 50:438-447, 2002). There is a need for a cotton plant more highly glyphosate tolerant than Roundup Ready® cotton 1445.

[0004] It would be advantageous to be able to detect the presence of a particular event in order to determine whether the progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring pre-market approval or labeling of foods derived from recombinant crop

plants, for example. It is possible to detect the presence of a transgene by any well known nucleic acid detection method such as the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, 3' transcription terminators, marker genes, etc. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct unless the sequence of genomic chromosomal DNA adjacent to the inserted DNA ("flanking genomic DNA") is known. Event-specific DNA detection methods for a glyphosate tolerant cotton event 1445 have been described (US 20020120964, herein incorporated by reference in its entirety).

[0005] The present invention relates to a glyphosate tolerant cotton event MON 88913, compositions contained therein, and to the method for the detection of the transgene/genomic insertion region in cotton event MON 88913 and progeny thereof.

SUMMARY OF THE INVENTION

[0006] The present invention is related to the transgenic cotton event designated MON 88913 having seed deposited with American Type Culture Collection (ATCC) with Accession No. PTA-4854. Another aspect of the invention comprises the progeny plants, or seeds, or regenerable parts of the plants and seeds of the cotton event MON 88913. The invention also includes plant parts of cotton event MON 88913 that include, but are not limited to pollen, ovule, flowers, bolls, lint, shoots, roots, and leaves. The invention relates to a cotton plant having a glyphosate tolerant phenotype and the novel genetic compositions of MON 88913.

[0007] One aspect of the invention provides DNA compositions and methods for detecting the presence of a transgene/genomic junction region from cotton plant event MON 88913. Isolated DNA molecules are provided that comprise at least one transgene/genomic junction DNA molecule selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, and complements thereof, wherein the junction molecule spans the insertion site that comprises a heterologous DNA inserted into the cotton genome and the genomic DNA from the cotton cell flanking the insertion site in cotton event MON 88913. A cottonseed and plant material thereof comprising these molecules is an aspect of this invention.

[0008] An isolated novel DNA molecule is provided that is a 5'transgene/genomic region SEQ ID NO:3 or the complement thereof, wherein this DNA molecule is novel in cotton event

MON 88913. A cotton plant and seed comprising SEQ ID NO:3 in its genome is an aspect of this invention. According to another aspect of the invention, an isolated DNA molecule is provided that is a 3'transgene/genomic region SEQ ID NO:4, or the complement thereof, wherein this DNA molecule is novel in cotton event MON 88913. A cotton plant and seed comprising SEQ ID NO:4 in its genome is an aspect of this invention.

[0009] According to another aspect of the invention, two DNA molecules are provided for use in a DNA amplification method, wherein the first DNA molecule comprises at least 11 or more contiguous polynucleotides of any portion of the transgene region of the DNA molecule of SEQ ID NO:3 and a DNA molecule of similar length of any portion of a 5' flanking cotton genomic DNA region of SEQ ID NO:3, where these DNA molecules when used together are useful as a DNA primer set in a DNA amplification method that produces an amplicon. The amplicon produced using the DNA primer set in the DNA amplification method is diagnostic for cotton event MON 88913. Any amplicon produced from MON 88913 DNA by DNA primers that are homologous or complementary to any portion of SEQ ID NO:3 is an aspect of the invention.

[0010] According to another aspect of the invention, two DNA molecules are provided for use in a DNA amplification method, wherein the first DNA molecule comprises at least 11 or more contiguous polynucleotides of any portion of the transgene region of the DNA molecule of SEQ ID NO:4 and a DNA molecule of similar length of any portion of a 3' flanking cotton genomic DNA of SEQ ID NO:4, where these DNA molecules are useful as a DNA primer set in a DNA amplification method. The amplicon produced using the DNA primer set in the DNA amplification method is diagnostic for cotton event MON 88913. The amplicons produced from MON 88913 DNA by DNA primers that are homologous or complementary to any portion of SEQ ID NO:4 are an aspect of the invention.

[0011] According to another aspect of the invention, methods of detecting the presence of DNA corresponding specifically to the cotton event MON 88913 DNA in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set that, when used in a nucleic acid amplification reaction with genomic DNA from cotton event MON 88913 produces an amplicon that is diagnostic for cotton event MON 88913

(b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

[0012] According to another aspect of the invention, methods of detecting the presence of DNA corresponding specifically to the cotton event MON 88913 DNA in a sample are provided. Such methods comprising: (a) contacting the sample comprising DNA with a DNA probe comprising SEQ ID NO:1 or SEQ ID NO:2, that hybridize under stringent hybridization conditions with genomic DNA from cotton event MON 88913 and does not hybridize under the stringent hybridization conditions with a control cotton plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the cotton event MON 88913 DNA.

[0013] According to another aspect of the invention, methods of producing a cotton plant that tolerates application of glyphosate are provided that comprise the steps of: (a) sexually crossing a first parental cotton event MON 88913 comprising the expression cassettes of the present invention, which confers tolerance to application of glyphosate, and a second parental cotton plant that lacks the glyphosate tolerance, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that tolerates application of glyphosate. Such methods may optionally comprise the further step of backcrossing the progeny plant to the second parental cotton plant and selecting for glyphosate tolerant progeny to produce a true-breeding cotton variety that tolerates application of glyphosate.

[0014] According to another aspect of the invention, a method is provided for determining the zygosity of the progeny of cotton event MON 88913 comprising:(a) contacting the sample comprising cotton DNA with a primer set comprising SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25 that when used in a nucleic-acid amplification reaction with genomic DNA from cotton event MON 88913, produces a first amplicon that is diagnostic for cotton event MON 88913; and (b) performing a nucleic acid amplification reaction, thereby producing the first amplicon; and (c) and detecting the first amplicon; and (d) contacting the sample comprising cotton DNA with said primer set, that when used in a nucleic-acid amplification reaction with genomic DNA from cotton plants produces a second amplicon comprising the native cotton genomic DNA homologous to the cotton genomic region of a transgene insertion identified as cotton event MON 88913; and (e) performing a

nucleic acid amplification reaction, thereby producing the second amplicon; and (f) and detecting the second amplicon; and (g) comparing the first and second amplicons in a sample, wherein the presence of both amplicons indicates the sample is heterozygous for the transgene insertion.

[0015] A method for determining zygosity comprising contacting a cotton DNA sample with using with primers and probes comprising SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; using an endpoint Taqman® PCR condition; and detecting the amplicon products.

[0016] A method for controlling weeds in a crop or field of cotton event MON 88913 comprising the step of applying a herbicidally effective amount of glyphosate containing herbicide to the field of MON 88913 cotton.

[0017] The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1. Plasmid map of pMON51915.

[0019] Figure 2. Genomic organization of insert in cotton event MON 88913.

[0020] Figure 3. MON 88913 5' DNA junction sequence (SEQ ID NO:1) and 3' DNA junction sequence (SEQ ID NO:2).

[0021] Figure 4. MON 88913 5' transgene/genomic DNA region (SEQ ID NO:3).

[0022] Figure 5. MON 88913 3' transgene/genomic DNA region (SEQ ID NO:4).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0023] The present invention relates to a glyphosate tolerant cotton event MON 88913, compositions contained therein, and to the method for the detection of the transgene/genomic insertion region in cotton event MON 88913 and progeny thereof. The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms and abbreviations are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger *et al.*,

Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York, 1991; and Lewin, *Genes V*, Oxford University Press: New York, 1994. The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used.

[0024] As used herein, the term "cotton" means *Gossypium hirsutum* and includes all plant varieties that can be bred with cotton event MON 88913. The plant of the present invention is a cotton plant, more specifically the cotton plant MON 88913.

[0025] As used herein, the term "comprising" means "including but not limited to".

[0026] As used herein, the term "crop" refers to cultivated plants or parts of plants, such as are grown in a field, plot, row, greenhouse, flat, or container.

[0027] "Glyphosate" refers to N-phosphonomethylglycine and its salts. N-phosphonomethylglycine is a well-known herbicide that has activity on a broad spectrum of plant species. Glyphosate is the active ingredient of Roundup® (Monsanto Co.), a safe herbicide having a desirably short half-life in the environment. Glyphosate is the active ingredient of Roundup® herbicide (Monsanto Co.). Treatments with "glyphosate herbicide" refer to treatments with the Roundup®, Roundup Ultra®, Roundup Pro® herbicide or any other herbicide formulation containing glyphosate. Examples of commercial formulations of glyphosate include, without restriction, those sold by Monsanto Company as ROUNDUP®, ROUNDUP® ULTRA, ROUNDUP® ULTRAMAX, ROUNDUP® WEATHERMAX, ROUNDUP® CT, ROUNDUP® EXTRA, ROUNDUP® BIACTIVE, ROUNDUP® BIOFORCE, RODEO®, POLARIS®, SPARK® and ACCORD® herbicides, all of which contain glyphosate as its isopropylammonium salt; those sold by Monsanto Company as ROUNDUP® DRY and RIVAL® herbicides, which contain glyphosate as its ammonium salt; that sold by Monsanto Company as ROUNDUP® GEOFORCE, which contains glyphosate as its sodium salt; and that sold by Syngenta Crop Protection as TOUCHDOWN® herbicide, which contains glyphosate as its trimethylsulfonium salt. When applied to a plant surface, glyphosate moves systemically through the plant. Glyphosate is phytotoxic due to its inhibition of the shikimic acid pathway, which provides a precursor for the synthesis of aromatic amino acids. Glyphosate inhibits the enzyme 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS) found in plants. Glyphosate tolerance can be achieved by the expression of bacterial EPSPS variants and plant EPSPS variants that have lower affinity for glyphosate and therefore retain their catalytic

activity in the presence of glyphosate (U.S. Patent Nos. 5,633,435, 5,094,945, 4,535,060, and 6,040,497).

[0028] A transgenic "event" is produced by transformation of a plant cell with heterologous DNA, *e.g.*, a nucleic acid construct (pMON51915, Figure 1) that includes a transgene of interest; regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant cell, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant plant and progeny of the transformant that include the heterologous DNA. The term "event" also includes progeny produced by a sexual outcross between the event and another plant that wherein the progeny includes the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking genomic DNA from the transformed parent event is present in the progeny of the cross at the same chromosomal location. The term "event" also refers to DNA from the original transformant comprising the inserted DNA, and flanking genomic sequence immediately adjacent to the inserted DNA, that would be expected to be transferred to a progeny that receives the inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (*e.g.*, the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

[0029] The expression of foreign genes in plants is known to be influenced by their chromosomal position, perhaps due to chromatin structure (*e.g.*, heterochromatin) or the proximity of transcriptional regulation elements (*e.g.*, enhancers) close to the integration site (Weising *et al.*, Ann. Rev. Genet 22:421-477, 1988). For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of a introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced transgene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression

levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual crossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions and market demands.

[0030] A glyphosate tolerant cotton plant can be bred by first sexually crossing a first parental cotton plant, consisting of a cotton plant grown from the transgenic cotton plant cell derived from transformation with the plant expression cassettes contained in pMON51915 and that tolerates application of glyphosate herbicide, with a second parental cotton plant that lacks the tolerance to glyphosate herbicide, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is tolerant to glyphosate herbicide; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants a glyphosate herbicide tolerant plant. These steps can further include the back-crossing of the first glyphosate tolerant progeny plant or the second glyphosate tolerant progeny plant to the second parental cotton plant or a third parental cotton plant, thereby producing a cotton plant that tolerates the application of glyphosate herbicide. In the present invention, the transgenic cotton plant is also defined as cotton event MON 88913 and may be referred to herein as MON 88913.

[0031] It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, *e.g.*, Fehr, in *Breeding Methods for Cultivar Development*, Wilcox J. ed., American Society of Agronomy, Madison WI (1987).

[0032] A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, *e.g.*, a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the

present invention, to a strand of genomic DNA from MON 88913 whether from a MON 88913 plant or from a sample that includes MON 88913 DNA. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

[0033] DNA primers are isolated polynucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, *e.g.*, a DNA polymerase. A DNA primer pair or a DNA primer set of the present invention refer to at least two DNA primer molecules useful for amplification of a target nucleic acid sequence, *e.g.*, by the polymerase chain reaction (PCR) or other conventional nucleic acid amplification methods.

[0034] Probes and primers are generally 11 polynucleotides or more in length, often 18 polynucleotides or more, 24 polynucleotides or more, or 30 polynucleotides or more. Such probes and primers are selected to be of sufficient length to hybridize specifically to a target sequence under high stringency hybridization conditions. Preferably, probes and primers according to the present invention have complete sequence similarity with the target sequence, although probes differing from the target sequence that retain the ability to hybridize to target sequences may be designed by conventional methods.

[0035] Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 (hereinafter, "Sambrook *et al.*, 1989"); *Current Protocols in Molecular Biology*, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel *et al.*, 1992"); and Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR DNA primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

[0036] Primers and probes based on the flanking genomic DNA and transgene insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed

DNA sequences by conventional methods, *e.g.*, by isolation of genomic DNA from MON 88913, re-cloning the transgene/genomic regions and sequencing such DNA molecules.

[0037] The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA molecule. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Polynucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two polynucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook *et al.*, 1989, and by Haymes *et al.*, In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

[0038] As used herein, a substantially homologous DNA sequence is the sequence of a DNA molecule that will specifically hybridize to the complement of a target DNA molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions that promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989),

BNSDOCID: <WO 2004072235A2 >

6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. In a preferred embodiment, a polynucleic acid of the present invention will specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NO: 3 or 4, or complements thereof or fragments of either under moderately stringent conditions, for example at about 2.0 x SSC and about 65°C. In a particularly preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NO:3 or 4 or complements or fragments of either under high stringency conditions. In one aspect of the present invention, a preferred marker nucleic acid molecule of the present invention has the nucleic acid sequence set forth in SEQ ID NO:1 or 2 or complements thereof or fragments of either. In another aspect of the present invention, a preferred marker nucleic acid molecule of the present invention shares a substantial portion of its sequence identity with the nucleic acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2 or complement thereof or fragments of either, wherein the sequence identity is between 80% and 100% or 90% and 100%. In a further aspect of the present invention, a preferred marker nucleic acid molecule of the present invention shares between 95% and 100% sequence identity with the sequence set forth in SEQ ID NO:1 or SEQ ID NO:2 or complement thereof or fragments of either. SEQ ID NO:1 or SEQ ID NO:2 may be used as markers in plant breeding methods to identify the progeny of genetic crosses similar to the methods described for simple sequence repeat DNA marker analysis, in "DNA markers: Protocols, applications, and overviews: (1997) 173-185, Cregan, *et al.*, eds., Wiley-Liss NY; herein incorporated by reference in its entirely. The hybridization of the probe to the target DNA molecule can be detected by any number of methods known to those skilled in the art, these can include, but are not limited to, fluorescent tags, radioactive tags, antibody based tags, and chemiluminescent tags.

[0039] Regarding the amplification of a target nucleic acid sequence (*e.g.*, by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the

primer pair to hybridize only to the target nucleic acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

[0040] The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

[0041] As used herein, "amplified DNA" or "amplicon" refers to the product of polynucleic acid amplification method directed to a target polynucleic acid molecule that is part of a polynucleic acid template. For example, to determine whether a cotton plant resulting from a sexual cross contains transgenic event genomic DNA from the cotton event MON 88913 plant of the present invention, DNA that is extracted from a cotton plant tissue sample may be subjected to a polynucleic acid amplification method using a primer pair that includes a primer derived from DNA sequence in the genome of the MON 88913 plant adjacent to the insertion site of the inserted heterologous DNA (transgene DNA), and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the MON 88913 event DNA. The diagnostic amplicon is of a length and has a DNA sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair, preferably plus about fifty nucleotide base pairs (bps), more preferably plus about two hundred-fifty nucleotide base pairs, and even more preferably plus about four hundred-fifty nucleotide base pairs or more. Alternatively, a primer pair can be derived from genomic sequence on both sides of the inserted heterologous DNA so as to produce an amplicon that includes the entire insert polynucleotide sequence (*e.g.*, a forward primer isolated from the genomic portion of SEQ ID NO:3 and a reverse primer isolated from the genomic portion of SEQ ID NO:4 that amplifies a DNA molecule comprising the two expression cassettes of pMON51915 DNA fragment that was inserted into the MON 88913 genome, the insert comprising about 8,512 bps of the insert, Figure 2). A member of a primer pair derived from the plant genomic sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to about twenty thousand nucleotide base pairs. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

[0042] Polynucleic acid amplification can be accomplished by any of the various polynucleic acid amplification methods known in the art, including the polymerase chain reaction (PCR). Amplification methods are known in the art and are described, *inter alia*, in U.S. Patent Nos. 4,683,195 and 4,683,202 and in *PCR Protocols: A Guide to Methods and Applications*, ed. Innis *et al.*, Academic Press, San Diego, 1990. PCR amplification methods have been developed to amplify up to 22 kb (kilobase) of genomic DNA and up to 42 kb of bacteriophage DNA (Cheng *et al.*, Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. The sequence of the heterologous DNA insert or flanking genomic DNA sequence from MON 88913 can be verified (and corrected if necessary) by amplifying such DNA molecules from the MON 88913 seed or plants grown from the seed deposited with the ATCC having accession no. PTA-4854, using primers derived from the sequences provided herein, followed by standard DNA sequencing of the PCR amplicon or cloned DNA fragments thereof. DNA detection kits that are based on DNA amplification methods contain DNA primers that specifically amplify a diagnostic amplicon. The kit may provide an agarose gel based detection method, endpoint Taqman®, or any number of methods of detecting the diagnostic amplicon that are known in the art. A kit that contains DNA primers that are homologous or complementary to any portion of SEQ ID NO:3 or SEQ ID NO:4 is an object of the invention.

[0043] The amplicon produced by these methods may be detected by a plurality of techniques. One such method is Genetic Bit Analysis (Nikiforov, *et al.* Nucleic Acid Res. 22:4167-4175, 1994) where a DNA oligonucleotide is designed that overlaps both the adjacent flanking genomic DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microtiter plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking genomic sequence), a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labelled dideoxynucleotide triphosphates (ddNTPs) specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the transgene/genomic sequence due to successful amplification, hybridization, and single base extension.

[0044] Another method is the Pyrosequencing technique as described by Winge (Innov. Pharma. Tech. 00:18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. Deoxynucleotide triphosphates (dNTPs) are added individually and the incorporation results in a light signal that is measured. A light signal indicates the presence of the transgene/genomic sequence due to successful amplification, hybridization, and single or multi-base extension.

[0045] Fluorescence Polarization as described by Chen, *et al.*, (Genome Res. 9:492-498, 1999) is a method that can be used to detect the amplicon of the present invention. Using this method an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene/genomic sequence due to successful amplification, hybridization, and single base extension.

[0046] Taqman® (PE Applied Biosystems, Foster City, CA) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the genomic flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the transgene/genomic sequence due to successful amplification and hybridization.

[0047] Molecular Beacons have been described for use in sequence detection as described in Tyangi, *et al.* (Nature Biotech.14:303-308, 1996) Briefly, a FRET oligonucleotide

probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

[0048] DNA detection kits can be developed using the compositions disclosed herein and the methods well known in the art of DNA detection. The kits are useful for identification of cotton event MON 88913 DNA in a sample and can be applied to methods for breeding cotton plants containing MON 88913 DNA. The kits contain DNA sequences that are useful as primers or probes and that are homologous or complementary to any portion of SEQ ID NO:3 or SEQ ID NO:4 or to DNA sequences homologous or complementary to DNA contained in any of the transgene genetic elements of pMON51915 that have been inserted into MON 88913 DNA (Figure 2). These DNA sequences can be used in DNA amplification methods (PCR) or as probes in polynucleic acid hybridization methods, *i.e.*, Southern analysis, northern analysis. The transgene genetic elements contained in MON 88913 DNA (Figure 2) include a first expression cassette comprising the Figwort mosaic promoter constructed as a chimeric promoter element with the *Arabidopsis* elongation factor 1-alpha (At.Ef1α) promoter (FMV35S/Eflα, U.S. Patent 6,462,258, SEQ ID NO:28, herein incorporated by reference in its entirely), operably linked to the *Arabidopsis* elongation factor 1-alpha translational leader and intron (Genbank accession number X16430 as described in Axelos *et al.*, *Mol. Gen. Genet.* 219:106-112, 1989), operably linked to the *Arabidopsis* EPSPS chloroplast transit peptide (TS-At.EPSPS:CTP2, Klee *et al.*, Mol. Gen. Genet. 210:47-442, 1987), operably linked to a glyphosate tolerant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (aroA:CP4, U.S. Patent 5,633,435), operably linked to the 3' termination region from pea ribulose 1,5-bisphosphate carboxylase E9 (T-Ps.RbcS2:E9, Coruzzi, *et al.*, EMBO J. 3:1671-1679, 1984), and a second expression cassette comprising the CaMV35S-Act8 promoter including the first intron

of the Act8 gene (SEQ ID NO:29, U.S. Patent 6,462,258) operably connected to an *Arabidopsis* EPSPS chloroplast transit peptide (TS-At.EPSPS:CTP2), operably connected to a glyphosate tolerant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (aroA:CP4, U.S. Patent 5,633,435, herein incorporated by reference in its entirety), operably linked to the 3' termination region from pea ribulose 1,5-bisphosphate carboxylase E9.

[0049] The following examples are included to demonstrate examples of certain preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1

[0050] The transgenic cotton event MON 88913 was generated by an *Agrobacterium*-mediated transformation of cotton cells with a DNA fragment derived from pMON51915 (Figure 1). The plant transformation construct, pMON51915 was mated into *Agrobacterium* using a triparental mating procedure (Ditta *et al.*, Proc. Natl. Acad. Sci. 77:7347-7351, 1980). Cotton cell transformation with transgenes can be performed using methods described, *e.g.*, in U.S. Patent 5,004,863, U.S. Patent 5,159,135, and U.S. Patent 5,518,908, herein incorporated by reference in their entirety. Cotton transformation is performed essentially as described in WO/0036911 or as described in U.S. Patent 5,846,797, herein incorporated by reference in its entirety. A modification of these methods can include, but is not limited to the following example. Coker 130 seed is surface sterilized and germinated in the dark. Hypocotyl explants are cut from the germinated seedlings to lengths of about 1-1.5 centimeter. *Agrobacterium tumefaciens* strain ABI transformed to contain pMON51915 is grown in Luria broth without antibiotics for 16 hours at 28 °C, then diluted to approximately 2 X 10^8 bacteria/milliter (ml). The hypotocyl explant is submersed in the *Agrobacterium* inoculum for 2-5 minutes, then co-cultivated for about 45 hours on MS+1.9mg/l KNO3+3%glucose (TRM),30 explants per plate,

24C, in the dark. The explants are transferred to TRM containing 150 mg/l cefotaxime and 300 EM glyphosate for four culture periods, each period for approximately six weeks. Embryogenic calli is segregated from the primary explant at the end of 3rd or 4th culture periods and placed onto same medium. The embryogenic calli are subcultured once by briefly suspending in liquid TRM +3% glucose, followed by pouring suspension onto 'TRM' + 150mg/l cefotaxime + 300,uM glyphosate plates. The somatic embryos are harvested 3-8 weeks after the liquid subculture, then grown on Stewart and Hsu media with 0. 5% glucose. Plantlets derived from the somatic embryos are matured to about 4-7cm (3-6 leaves) in Magenta boxes with Stewart & Hsu modified with 40mM NO3/lOmM NH4 + 2% sucrose. These plants are then transplanted to potting soil, 4" pots, 100% humidity, 16hours of light per day, for 4- 6 days, followed by 50% humidity 5-10 days.

[0051] The DNA fragment of pMON51915 contains two transgene expression cassettes inserted into the genome of MON 88913 (Figure 2) that collectively confer glyphosate tolerance to MON 88913 and progeny thereof.

[0052] The MON 88913 plant and seed has regenerable parts. The regenerable parts of the seed include, but are not limited to the embryo, the cotyledon, and the shoot or root meristem. The regenerable parts of the plant include, but are not limited to the leaves, the petiole, the hypocotyl, stem sections, and apical and root meristems. The invention also includes plant parts of cotton event MON 88913 that include, but are not limited to pollen, ovule, flowers, bolls, lint, shoots, roots, and leaves. The invention also includes extractable components of MON 88913 seed that include, but are not limited to protein, meal, flour, hulls, oil, and linter.

EXAMPLE 2

[0053] The glyphosate tolerant cotton event MON 88913 was selected from many transgenic cotton events for tolerance to glyphosate vegetative and reproductive injury. The successful production of a commercial quality transgenic event currently requires producing a large number of transgenic events. In the present invention, MON 88913 was one event among approximately 1000 R0 events that had been transformed with many different DNA constructs that included pMON51915. The MON 88913 event was selected from the many events by a series of molecular analysis and glyphosate tolerance screens.

[0054] The events were screened in a greenhouse glyphosate tolerance test, the plants being scored for vegetative and reproductive tolerance. Fifteen to twenty-five R_1 seeds from each event were planted in 15 cell trays with Metro-Mix 350 growing medium, which contains a combination of peat, vermiculite, nutrients, wetting agents, and processed bark and ash. Additional fertilizers included in the medium were Osmocote 14-14-14, Osmocote Plus 15-9-12, and MicroMax micronutrients. All plants were grown in a greenhouse. The average daytime temperature during the growing season was 32 degrees Celsius (°C), while the average night temperature was 24°C. The photoperiod was set at 16 hours of light and eight hours of dark, with maximum light intensity. The average relative humidity during the growth cycle was 45 percent. The plants were then sprayed at the 4 and 8-leaf stages sequentially with 48 oz/A (oz=ounces, A=acre) of Roundup Ultra® (glyphosate containing herbicide). Seven days after the 4-leaf glyphosate application, the plants were scored for vegetative damage and segregation of the glyphosate tolerant phenotype was collected. These data were used to confirm that the event transgene insert was performing as a single dominant gene, adhering to Mendelian genetic models. Events with good vegetative tolerance were subsequently transplanted in 10-inch pots with the same Metro-Mix 350 growing medium described above and grown to maturity. Plants were treated with Pix Plus (BASF, Research Triangle Park, NC) as needed to regulate the plant height. At three months post-planting, the plants were mapped for boll retention on the first fruiting positions of the first five fruiting branches. The maximum value for retention for this plant map is 5 (five bolls retained). This provided a quick, indirect measure of plant fertility. With this greenhouse screen, the average retention for the current commercial event (RR cotton 1445) is less than 1.0. Events with an average boll retention value greater than or equal to three were harvested and advanced for further event selection. Events that have boll retention values greater than or equal to two have value as new glyphosate tolerant plant selections.

[0055] Events that met the Roundup® Ultra vegetative and reproductive tolerance criteria were analyzed for copy number via Southern blot analysis. Single copy events that showed good tolerance in initial greenhouse experiments were further characterized in 1) additional greenhouse tolerance tests at higher glyphosate rates, 2) replicated field trials, and with 3) additional molecular screens. The greenhouse tolerance tests were conducted using homozygous plants. All of the experiments contained the current commercial Roundup Ready®

cotton event 1445 event (commercial standard) for comparison. Seed were planted in 15 cell trays and treated with 64oz/A Roundup Ultra® at the 4-leaf stage and 96oz/A at the 8-leaf stage. The plants were then transplanted to 10-inch pots and four plants were mapped at mid-season on the first fruiting positions of the first five fruiting branches. End of the season data were also collected on all events and included seed cotton weight, number of bolls, boll size, and boll retention.

[0056] Field testing was used to select the event that showed best growth rates, fruit retention, and yield. The field trials were arranged in a randomized split plot design with three replications and three treatments. The events were planted in two row, 30-foot plots. The treatments consisted of unsprayed, 64oz/A (1.5 lb ae/A) Roundup Ultra® at the 4, 6, 10, 14-node stages, and 96oz/A (2.25 lb ae/A) Roundup Ultra® at the 4, 6, 10, 14-node stages. A mid-season plant map was completed on ten plants per plot. Boll retention data was collected for the first and second fruiting positions of the first five fruiting nodes that provides a boll retention value scale of 0-10. A plant with a boll retention value equal to or greater than 3 on the 0-10 scale has value as a new glyphosate tolerant plant selection.

[0057] Field testing (10 locations) comparing the yield of cotton lint (pounds/acre, lb/A) from MON 88913 and RR cotton 1445 showed that MON 88913 provided substantial protection against glyphosate (pounds of acid equivalent/acre, lb ae/A) effects on yield (Table 1). Yield is a measure of boll retention, cotton plants engineered for glyphosate tolerance that retain a substantial number of bolls in the first and second fruiting positions will maintain a yield advantage over cotton plants that are not as glyphosate tolerant. An effective dose of a glyphosate containing herbicide to control weeds in a field of MON 88913 comprises about 4 oz/A and may exceed 128 oz/A depending on the species of weed to be controlled and the stage of weed development. Glyphosate can be mixed with other herbicides to enhance the herbicidal activity against certain weed species.

Table 1. Comparison of lint yield of MON 88913 and 1445 after glyphosate treatment.

	Yield (lb/A) of 10 Locations		
Glyphosate treatment	0 lb ae/A	1.5 lb ae/A	2.25 lb ae/A
1445	2421.84	1044.19	831.47
MON 88913	2551.58	2587.61	2412.3

EXAMPLE 3

[0058] Cotton genomic DNA for all PCR reactions and Southern blot analyses was isolated using a CTAB procedure (Rogers *et al.*, Plant Mol. Biol. 5:69-76, 1985) or Dneasy™ 96 Plant Kit (Cat. # 69181, Qiagen Inc., Valencia, CA) following the manufacturers instructions. Leaf tissue was collected from plants at the 2-4-leaf stage. The smallest true leaves were collected from each plant and immediately frozen on dry ice. DNA was extracted using, *e.g.*, the following method. The tissue was ground using plastic beads with liquid nitrogen. Five ml of extraction buffer was added to 0.75 gram (g) of tissue and incubated at 55°C for 45 minutes. The CTAB extraction buffer consisted of 100mM Tris pH8.0, 1.4M NaCl, 20mM EDTA, 2% CTAB with the addition of 5µl (microliter) of beta-mercaptoethanol, 5µl of RNase and 1% PVPP. The samples were then extracted with an equal volume of chloroform (5ml) and then centrifuged at 3700 RPM for 15 minutes at room temperature. The aqueous phase was transferred to a new tube and the DNA was precipitated with an equal volume of isopropanol. After centrifugation at 3700 RPM for 15 minutes, the pellets were washed with 70% ethanol, air dried, and resuspended in 250µl of water.

[0059] Cotton genomic DNA adjacent to the transgene insertion was obtained for the MON 88913 event utilizing TAIL-PCR (Liu *et al.*, Plant Journal 8: 457-463, 1995). Extension of the genomic DNA was conducted using the GenomeWalker kit (CloneTech Laboratories, Palo Alto, CA) following the manufacture's protocol. Briefly, the DNA (~5µg) isolated utilizing the CTAB protocol previously described, was digested with various restriction endonucleases (EcoRV, ScaI) at 37°C overnight in a total volume of 100µl. The restriction endonucleases were removed with QIAquick PCR Purification columns (cat #28104, Qiagen, Inc.). The ligation of adaptor molecules were those that were described in the manufacture's protocol.

BNSDOCID: <WO 2004072235A2 >

DNA was amplified using FMV-1 primer (SEQ ID NO:5) with the AP1 primer (CloneTech Laboratories) for the primary reaction and nested FMV-2 primer (SEQ ID NO:6) with the AP2 primer (CloneTech Laboratories) for the secondary reaction.

[0060] The 3' transgene/genomic DNA of the MON 88913 was isolated utilizing inverse PCR. Total genomic DNA (~10µg) was digested with three restriction enzymes; *Bcl*I, *Nco*I, and *Hind*III. The QIAquick PCR Purification columns were used to purify the DNA after digesting overnight at 37°C. The DNA was eluted from the columns with 50µl of water and then diluted to 1ml. The diluted eluate (85µl) was combined with 10µl of buffer (10X) and 5µl of T4 Ligase to circularize the fragments. After an overnight incubation at 16°C, the ligase was heat inactivated at 70°C. The samples were amplified by PCR with a series of nested primers. The primer combinations for PCR included: primary pair 8099-E9-1/E9-2 (SEQ ID NO:7/SEQ ID NO:8) for *Bcl*I and *Nco*I samples and primer pair 8099-E9-1/Act8 rev (SEQ ID NO:7/SEQ ID NO:9) for the *Hind*III sample; primer pair 8099-E9-2/E9-1 (SEQ ID NO:10/SEQ ID NO:11) for *Bcl*I and *Nco*I samples; primer pair 8099-E9-2/Act8 (SEQ ID NO:10/SEQ ID NO:12) for the *Hind*III sample; primer pair 8099-E9-3/E9-1 (SEQ ID NO:13/SEQ ID NO:11) for *Bcl*I and *Nco*I samples and 8099-E9-3/Act8 (SEQ ID NO:13/SEQ ID NO:12) for the *Hind*III sample. The conditions for the PCR included: primary PCR = 7 cycles of 94°C for 2 seconds, 72°C for 10 minutes; 37 cycles of 94°C for 2 seconds, 67°C for 10 minutes; 1 cycle of 67°C for 10 minutes; secondary and tertiary PCR = 5 cycles of 94°C for 2 seconds, 72°C for 10 minutes; 24 cycles of 94°C for 2 seconds, 67°C for 10 minutes; 1 cycle of 67°C for 10 minutes.

[0061] Alternatively, DNA amplification by PCR of the 3' end of the MON 88913 event can be performed with conditions that include: 7 cycles of 94°C for 25 seconds, 72°C for 3 minutes; 37 cycles of 94°C for 25 seconds, 67°C for 3 minutes; 1 cycle of 67°C for 7 minutes. All subsequent amplifications conducted with the following conditions: 7 cycles of 94°C for 2 seconds, 72°C for 4 minutes; 37 cycles of 94°C for 2 seconds, 67°C for 4 minutes; 1 cycle of 67°C for 7 minutes. All amplicons are visualized on 0.8% agarose gels stained with ethidium bromide. The DNA is prepared for sequencing either by purifying the PCR samples directly with the QIAquick PCR Purification kit (cat# 28104, Qiagen Inc.) or by extracting the appropriate fragment from the gel and using the QIAquick Gel Extraction kit (cat #28704, Qiagen Inc.).

[0062] A series of DNA primers were designed to sequence the transgene insert and the adjacent flanking genomic regions of the MON 88913. DNA primers were designed that allowed amplification of the entire transgene and genomic flanking regions by five overlapping fragments. Unique primers were designed to allow amplification of each EPSPS-CTP2/*aroA*-CP4/RbcS2:E9 region separately. For all fragments used in sequencing, the amplifications were performed in triplicate. The DNA primer pair combinations used as sequencing primers for the 5' transgene/genomic region (SEQ ID NO:14 and SEQ ID NO:15), 3' transgene/genomic region (SEQ ID NO:16 and SEQ ID NO:17) and insert genetic elements (SEQ ID NO:18 and SEQ ID NO:11; SEQ ID NO:19 and SEQ ID NO:15; SEQ ID NO:20 and SEQ ID NO:11). Total genomic DNA was used for all PCR reactions. All amplicons were visualized on 0.8% agarose gels stained with ethidium bromide. The DNA was prepared for sequencing either by purifying the PCR samples directly with the QIAquick PCR Purification kit or by extracting the appropriate fragment from the gel and using the QIAquick Gel Extraction kit. The DNA sequence was produced using DNA sequence analysis equipment (ABI Prism™ 377, PE Biosystems, Foster City, CA) and DNASTAR sequence analysis software (DNASTAR Inc., Madison, WI).

[0063] The DNA fragments from the flanking regions of MON 88913 transgene/genomic insert were subcloned using a TOPO TA Cloning® kit (Invitrogen). The DNA sequence of the 5' transgene/genomic region is shown in Figure 4 and the DNA sequence of the 3' transgene/genomic region is shown in Figure 5. In the DNA sequence shown in Figures 4 and 5, the transgene insert sequence is in italics.

EXAMPLE 4

[0064] DNA event primer pairs are used to produce an amplicon diagnostic for cotton event MON 88913 genome. Amplicons diagnostic for MON 88913 genome comprise at least one junction sequence, SEQ ID NO:1 or SEQ ID NO:2. Event primer pairs that will produce a diagnostic amplicon for MON 88913, in which the primer pairs include, but are not limited to, SEQ ID NO:14 and SEQ ID NO:15 for the 5' amplicon sequence, and SEQ ID NO:16 and SEQ ID NO:17 for the 3' amplicon when used in the protocol outlined in Table 2. In addition to these primer pairs, any primer pair, homologous or complementary to SEQ ID NO:3 or SEQ ID NO:4, that in a DNA amplification reaction produces an amplicon diagnostic for MON 88913 genome

is an aspect of the present invention. Any single isolated DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:3, or its complement that is useful in a DNA amplification method to produce an amplicon diagnostic for MON 88913 is an aspect of the invention. Any single isolated DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:4, or its complement that is useful in a DNA amplification method to produce an amplicon diagnostic for MON 88913 is an aspect of the invention. An example of the amplification conditions for this analysis is illustrated in Table 2 and Table 3, however, any modification of these methods that use DNA primers homologous or complementary to SEQ ID NO:3 or SEQ ID NO:4 or DNA sequences of the genetic elements contained in the transgene insert of MON 88913 that produce an amplicon diagnostic for MON 88913, is within the ordinary skill of the art. A diagnostic amplicon comprises a DNA molecule homologous or complementary to at least one transgene/genomic junction DNA (SEQ ID NO:1 or SEQ ID NO:2) or substantial portion thereof.

[0065] An analysis for event MON 88913 plant tissue sample should include a positive tissue control from event MON 88913, a negative control from a cotton plant that is not event MON 88913, and a negative control that contains no cotton genomic DNA. Additional primer sequences can be selected from SEQ ID NO:3 and SEQ ID NO:4 by those skilled in the art of DNA amplification methods, and conditions selected for the production of an amplicon by the methods shown in Table 2 and Table 3 may differ, but result in an amplicon diagnostic for event MON 88913. The use of these DNA primer sequences with modifications to the methods of Table 2 and 3 are within the scope of the invention. The amplicon produced by at least one DNA primer sequence derived from SEQ ID NO:3 or SEQ ID NO:4 that is diagnostic for MON 88913 is an aspect of the invention.

[0066] DNA detection kits that contain at least one DNA primer derived from SEQ ID NO:3 or SEQ ID NO:4 that when used in a DNA amplification method produces a diagnostic amplicon for MON 88913 is an aspect of the invention. The amplicon produced by at least one primer sequence derived from any of the genetic elements of pMON51915 that is diagnostic for MON 88913 is an aspect of the invention. A cotton plant or seed, wherein its genome will produce an amplicon comprising SEQ ID NO:1 or SEQ ID NO:2 when tested in a DNA amplification method is an aspect of the present invention. The assay for the MON 88913

amplicon can be performed by using a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler as shown in Table 3, or by methods and apparatus known to those skilled in the art.

Table 2. PCR procedure and reaction mixture conditions for the identification of MON 88913 5' transgene insert/genomic junction region.

Step	Reagent	Amount	Comments
1	Nuclease-free water	add to final volume of 20 µl	-
2	10X reaction buffer (with $MgCl_2$)	2.0 µl	1X final concentration of buffer, 1.5 mM final concentration of $MgCl_2$
3	10 mM solution of dATP, dCTP, dGTP, and dTTP	0.4 µl	200 µM final concentration of each dNTP
4	event primer (SEQ ID NO:14) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.4 µl	0.2 µM final concentration
5	event primer (SEQ ID NO:15) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.4 µl	0.2 µM final concentration
6	RNase, DNase free (500 ng/µl)	0.1 µl	50 ng/reaction

7	*REDTaq* DNA polymerase (1 unit/µl)	1.0 µl (recommended to switch pipets prior to next step)	1 unit/reaction
8	Extracted DNA (template): • Samples to be analyzed * individual leaves * pooled leaves (maximum of 50 leaves/pool) • Negative control • Negative control • Positive control	• 10-200 ng of genomic DNA • 200 ng of genomic DNA • 50 ng of cotton genomic DNA (not MON 88913) • no template DNA • 50 ng of MON 88913 genomic DNA	
9	Gently mix and add 1-2 drops of mineral oil on top of each reaction.		

Table 3. Suggested PCR parameters for different thermocyclers.

[0067] Proceed with the DNA amplification in a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler using the following cycling parameters. The MJ Engine or Eppendorf Mastercycler Gradient thermocycler should be run in the calculated mode. Run the Perkin-Elmer 9700 thermocycler with the ramp speed set at maximum.

Cycle No.	Settings: Stratagene Robocycler
1	94°C 3 minutes
38	94°C 1 minute 60°C 1 minute 72°C 1 minute and 30 seconds
1	72°C 10 minutes

Cycle No.	Settings: MJ Engine or Perkin-Elmer 9700
1	94°C 3 minutes
38	94°C 10 seconds 60°C 30 seconds 72°C 1 minute
1	72°C 10 minutes

Cycle No.	Settings: Eppendorf Mastercycler Gradient
1	94°C 3 minutes
38	94°C 15 seconds 60°C 15 seconds 72°C 1 minute and 30 seconds
1	72°C 10 minutes

EXAMPLE 5

[0068] MON 88913 genomic DNA and control cotton genomic DNA (~15µg of each) is digested with various restriction enzymes (140U) in a total volume of 150µl including 15µl of the corresponding manufacturer's buffer (NEB, Beverely, MA). Restriction endonucleases, *e.g.*, BglI, BamHI, NcoI, HindIII, and BclI, are used in the Southern analysis of MON 88913. Endonuclease digests are performed at the appropriate temperature for at least 6 hours. After incubating, the DNA is precipitated with 3M sodium acetate and 2.5 volumes of ethanol. Subsequently, the DNA is washed with 70% ethanol, dried, and resuspended in 40µl of TBE. Loading buffer (0.2x) is added to the samples and then electrophoresis conducted on agarose

gels (0.8%) for 16-18 hours at 30 volts. The gels are stained with ethidium-bromide, then treated with a depurination solution (0.125N HCL) for 10 minutes, with a denaturing solution (0.5M sodium hydroxide, 1.5M sodium chloride) for 30 minutes, and finally with a neutralizing solution (0.5M Trizma base, 1.5M sodium chloride) for 30 minutes. The DNA is transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) using a Turboblotter (Schleicher and Schuell, Dassel, Germany) for 4-6 hours and then fixed to the membrane using a UV light.

[0069] Membranes are prehybridized with 20mls of DIG Easy Hyb solution (Roche Molecular Biochemicals, Indianapolis, IN; cat. #1603558) for 2-4 hours at 45°C. Radioactive DNA probes (^{32}P dCTP) homologous or complementary to SEQ ID NO:1, or SEQ ID NO:2, or SEQ ID NO:3, or SEQ ID NO:4, or a portion thereof are made using a Radprime DNA Labeling kit (Invitrogen, Carlsbad, CA; cat. #18428-011). Unincorporated nucleotides are removed using sephadex G-50 columns (Invitrogen). The prehybridization solution is replaced with 10mls of pre-warmed DIG Easy Hyb solution containing the denatured probe to a final concentration of 1 million counts per ml. The blots are hybridized at 45°C for 16-18 hours.

[0070] Blots are washed with a low stringency solution (5X SSC, 0.1X SDS) at 45°C and then repeatedly washed with a higher stringency solution (0.1X SSC, 0.1% SDS) at 65°C. The blots are exposed to a phosphor screen (Amersham Biosciences, Piscataway, NJ) for >2 hours and the exposure read using a Data Storm 860 machine (Amersham Biosciences).

EXAMPLE 6

[0071] The methods used to identify heterozygous from homozygous cotton progeny containing event MON 88913 are described in a zygosity assay for which examples of conditions are described in Table 4 and Table 5. The DNA primers used in the zygosity assay are primer SQ1099 (SEQ ID NO:21), SQ1100 (SEQ ID NO:22), SQ1353 (SEQ ID NO:23), 6FAM™ labeled primer (SEQ ID NO:24,), and VIC™ labeled primer (SEQ ID NO:25), 6FAM and VIC are florescent dye products of Applied Biosystems (Foster City, CA) attached to the DNA primer.

[0072] SEQ ID NO:21, SEQ ID NO:22 and SEQ ID NO:23 when used in these reaction methods produce a DNA amplicon for non-transgenic cotton, two DNA amplicons for heterozygous cotton containing event MON 88913, and a DNA amplicon for homozygous MON

88913 cotton that is distinct from any other non–MON 88913 cotton. The controls for this analysis should include a positive control from homozygous and heterozygous cotton containing event MON 88913 DNA, a negative control from non-transgenic cotton, and a negative control that contains no template DNA. This assay is optimized for use with a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler. Other methods and apparatus known to those skilled in the art that produce amplicons that identify the zygosity of the progeny of crosses made with MON 88913 cotton plants is within the skill of the art.

Table 4. Zygosity assay reaction solutions

Step	Reagent	Amount	Comments
1	Nuclease-free water	add to 10 µl final volume	-
2	2X Universal Master Mix (Applied Biosystems cat. # 4304437)	5 µl	1 X final concentration
3	Primers SQ1099, SQ1100, SQ1353 (resuspended in nuclease-free water to a concentration of 20 µM)	0.5 µl	0.25 µM final concentration
4	Primer 6FAM™ (resuspended in nuclease-free water to a concentration of 10 µM)	0.2 µl	0.4 µM final concentration
5	Primer VIC™ (resuspended in nuclease-free water to a concentration of 10 µM)	0.2 µl	0.15 µM final concentration
6	*REDTaq* DNA polymerase (1 unit/µl)	1.0 µl (recommended to switch pipets prior to next step)	1 unit/reaction

7	Extracted DNA (template): • Samples to be analyzed (individual leaves) • Negative control • Negative control • Positive control • Positive control	3.0 µl • 4-80 ng of genomic DNA • 4 ng of non-transgenic cotton genomic DNA • no DNA template (solution in which DNA was resuspended) • 4 ng of genomic DNA from known event MON 88913 heterozygous cotton • 4 ng of genomic DNA from known event MON 88913 homozygous cotton	Diluted in water
8	Gently mix, add 1-2 drops of mineral oil on top of each reaction.		

Table 5. Zygosity assay thermocycler conditions

[0073] Proceed with the DNA amplificaition in a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler using the following cycling parameters. When running the PCR in the Eppendorf Mastercycler Gradient or MJ Engine, the thermocycler should be run in the calculated mode. When running the PCR in the Perkin-Elmer 9700, run the thermocycler with the ramp speed set at maximum.

Cycle No.	Settings: Stratagene Robocycler
1	94°C 3 minutes
38	94°C 1 minute 60°C 1 minute 72°C 1 minute and 30 seconds
1	72°C 10 minutes

Cycle No.	Settings: MJ Engine or Perkin-Elmer 9700
1	94°C 3 minutes
38	94°C 30 seconds 60°C 30 seconds 72°C 1 minute and 30 seconds
1	72°C 10 minutes

Cycle No.	Settings: Eppendorf Mastercycler Gradient
1	94°C 3 minutes
38	94°C 15 seconds 60°C 15 seconds 72°C 1 minute and 30 seconds
1	72°C 10 minutes

EXAMPLE 7

[0074] Analysis of cotton genomic DNA samples was conducted using an endpoint Taqman® method. The production of amplicons diagnostic for MON 88913 genomic DNA were produced by using a primer set A that included event primers: SEQ ID NO:21, SEQ ID NO:22, and 6-FAM probe SEQ ID NO:24; and a primer set B that included event primers: SEQ ID NO:26, SEQ ID NO:27, and 6-FAM probe SEQ ID NO:28. The method uses a 96-well or 384-well format and an Applied Biosystems GeneAmp PCR System 9700 or MJ Research DNA Engine PT-225. DNA extracted from cotton tissue samples as previously described should be within the range of 5-10 ng per PCR reaction. Each reaction contains a final volume of 10µl consisting of 0.5µl of equal concentration of the event primers (20µM), 5.0µl of 2X universal

master mix, 0.2µl of the 6-FAM probe (10µM), 3µl DNA sample (5-10 ng) and water to 10µl. The thermal cycler parameters are 1 cycle 50°C for 2 minutes, 1 cycle 95°C for 10 minutes, 10 cycles at 95°C for 15 seconds, 64°C for 1 minute then -1°C/cycle, 30 cycles 95°C for 15 seconds, 54°C for 1 minute, then hold at 10°C. The amplicon production was determined by a microplate reader, *e.g.*, a TECAN Safire (Durham, NC) using the conditions described by the manufacturer. A data analysis program (TaqPro™) was used to score the production of the labeled amplicon. Other equipment and analysis methods known in the art of DNA detection can be used to detect the amplicons of the present invention.

[0075] A deposit of Monsanto Technology LLC, cotton MON 88913 seed disclosed above and recited in the claims, has been made under the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110. The ATCC accession number is PTA-4854. The deposit will be maintained in the depository for a period of 30 years, or 5 years after the last request, or for the effective life of the patent, whichever is longer, and will be replaced as necessary during that period.

[0076] Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

[0077] All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS:

1. Seed of cotton event designated MON 88913 and having representative seed deposited with American Type Culture Collection (ATCC) with Accession No. PTA-4854.

2. The cotton plant or parts thereof produced by growing the seed of claim 1.

3. The cotton plant or parts thereof of claim 2, comprising pollen, ovule, flowers, bolls, lint, shoots, roots, or leaves.

4. Glyphosate tolerant progeny of the cotton plant of claim 2.

5. A progeny cotton plant of claim 4, wherein the genome of said cotton plant comprises one or more DNA molecules selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

6. A progeny cotton plant or seed or parts thereof of claim 4, the genome of which produces an amplicon comprising SEQ ID NO:1 or SEQ ID NO:2 in a DNA amplification method.

7. An isolated DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:3, or its complement that is useful in a DNA amplification method to produce an amplicon comprising SEQ ID NO:1 diagnostic for cotton event MON 88913.

8. An isolated DNA polynucleotide primer molecule comprising at least 11 contiguous nucleotides of SEQ ID NO:4, or its complement that is useful in a DNA amplification method to produce an amplicon comprising SEQ ID NO:2 diagnostic for cotton event MON 88913.

9. A DNA detection kit comprising at least one molecule of 11 or more contiguous nucleotides homologous or complementary to SEQ ID NO:3 or SEQ ID NO:4, that when used in a DNA amplification methods produces an amplicon comprising SEQ ID NO:1 or SEQ ID NO:2 diagnostic for cotton event MON 88913

10. A method of producing a cotton plant that tolerates application of glyphosate herbicide comprising:

(a) sexually crossing a first glyphosate tolerant cotton event MON 88913 parent plant and a second parent cotton plant that lacks the tolerance to glyphosate herbicide, thereby producing a plurality of first progeny plants; and

(b) selecting a first progeny plant that is tolerant to glyphosate; and

(c) selfing said first progeny plant, thereby producing a plurality of second progeny plants; and

(d) selecting from said second progeny plants, a glyphosate tolerant plant.

11. The method of claim 10 further comprising the step of backcrossing the first progeny plant that is tolerant to glyphosate or the second progeny plant that is glyphosate tolerant to the second parent plant or a third parent plant, thereby producing a plant that tolerates the application of glyphosate.

12. A method of detecting the presence of DNA corresponding to cotton event MON 88913 in a sample, the method comprising:

(a) contacting the sample comprising DNA with a DNA primer set, which when used in a nucleic acid amplification reaction with genomic DNA from the cotton event MON 88913, produces a diagnostic amplicon comprising SEQ ID NO:1 or SEQ ID NO:2; and

(b) performing a nucleic acid amplification reaction, thereby producing the diagnostic amplicon; and

(c) detecting the diagnostic amplicon.

13. In the method of claim 12, where in said primer set comprises SEQ ID NO:21, SEQ ID NO:22 and SEQ ID NO:24.

14. In the method of claim 12, wherein said primer set comprises SEQ ID NO:26, SEQ ID NO:27 and SEQ ID NO:28.

15. A method of detecting the presence of a DNA corresponding to cotton event MON 88913 in a sample, the method comprising:

(a) contacting the sample comprising DNA with a probe that hybridizes under stringent hybridization conditions with genomic DNA from the cotton event MON 88913 and does not hybridize under the stringent hybridization conditions with a control cotton plant genomic DNA, wherein said probe is homologous or complementary to SEQ ID NO:1 or SEQ ID NO:2; and

(b) subjecting the sample and probe to stringent hybridization conditions; and

(c) detecting hybridization of the probe to the DNA.

16. A cotton plant comprising a glyphosate tolerant trait that is genetically linked to a complement of a marker polynucleic acid, wherein said marker polynucleic acid molecule is homologous or complementary to a DNA molecule selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

17. A method of determining the zygosity of the progeny of cotton event MON 88913 comprising:

(a) contacting the sample comprising cotton DNA with a primer set comprising SEQ ID NO:21, SEQ ID NO:22 and SEQ ID NO:23, that when used in a nucleic-acid amplification reaction with genomic DNA from cotton event MON 88913, produces a first amplicon that is diagnostic for cotton event MON 88913; and

(b) performing a nucleic acid amplification reaction, thereby producing the first amplicon; and

(c) detecting the first amplicon; and

(d) contacting the sample comprising cotton DNA with said primer set, that when used in a nucleic-acid amplification reaction with genomic DNA from cotton plants produces a second amplicon comprising the native cotton genomic DNA homologous to the cotton genomic region of a transgene insertion identified as cotton event MON 88913;

(e) performing a nucleic acid amplification reaction, thereby producing the second amplicon; and

(f) detecting the second amplicon; and

(g) comparing the first and second amplicons in a sample, wherein the presence of both amplicons indicates the sample is heterozygous for the transgene insertion.

18. A method of determining the zygosity of the progeny of cotton event MON 88913 comprising:

(a) contacting the sample comprising cotton DNA with a primer set comprising SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, and SEQ ID NO:25; and

(b) performing a nucleic acid amplification reaction; and

(c) detecting the products of the reaction.

19. A method for controlling weeds in a crop of cotton event MON 88913 comprising the step of applying an effective dose of a glyphosate containing herbicide to said crop of cotton event MON 88913.

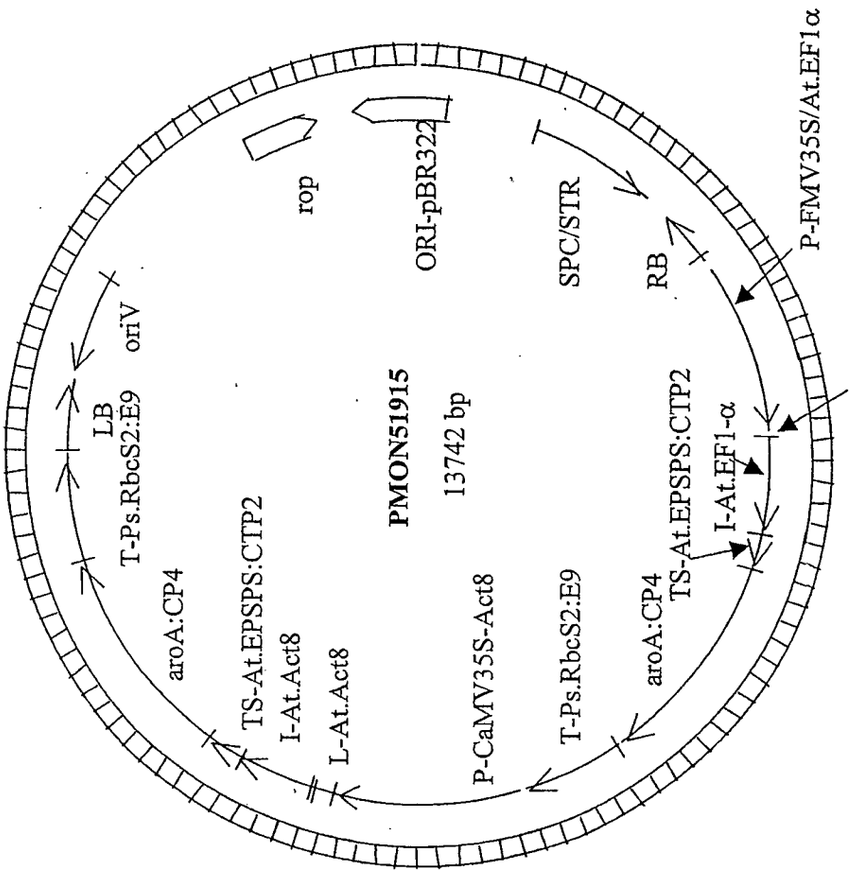

PMON51915
13742 bp
LB
T-Ps.RbcS2:E9
oriV
rop
ORI-pBR322
SPC/STR
RB
P-FMV35S/At.EF1α
L-At.EF1α
I-At.EF1-α
TS-At.EPSPS:CTP2
aroA:CP4
T-Ps.RbcS2:E9
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FIGURE 1

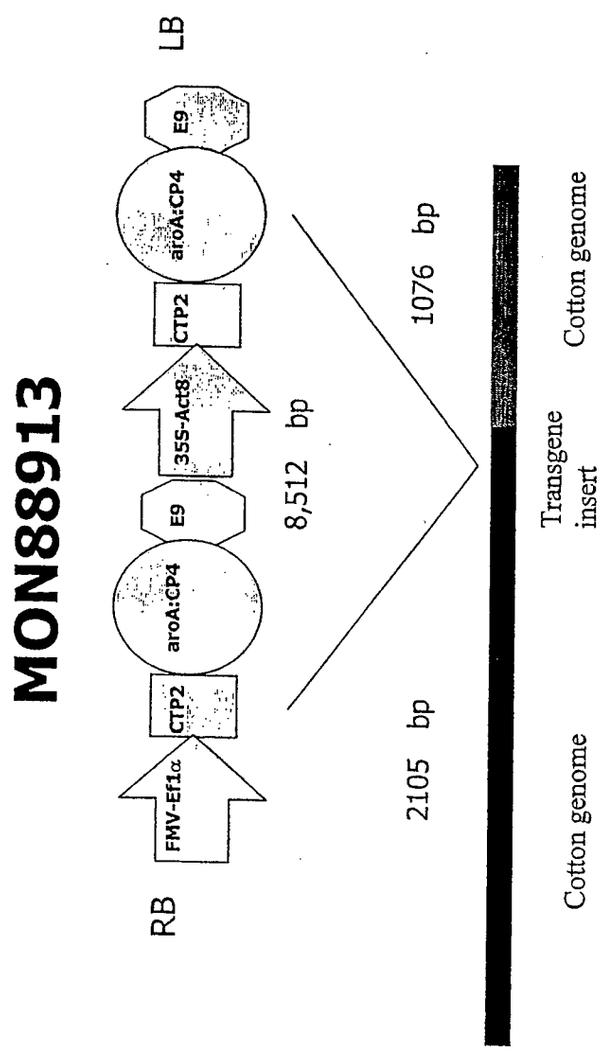

MON88913
RB
FMV-Ef1α
CTP2
aroA:CP4
E9
355-Act8
CTP2
aroA:CP4
E9
LB
8,512 bp
2105 bp
1076 bp
Cotton genome
Transgene insert
Cotton genome

FIGURE 2

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   1 GCTTGGTACC GAGCTCGGAT CCACTAGTAA CGGCCGCCAG TGTGCTGGAA TTCGCCCTTT
  61 TTTACTACGA TGTTAAGTCC TATTTTACAC AGTTTCTTTA AGACAGATTT GACCGCTCCT
 121 ACGATACTTG GAGAAACGTT GGTCGAATGT CTCTTAGAAT ACAACAACAC GATGATCAAA
 181 GCAGTAGCAC CTCTGTAGTG ATTAACGAAC AAGCGTTGTC TTTTTCTATC ACCAAAACAT
 241 TGGAAAACAT GGAGAGGAAA AGAGTAGAAT TTTGGAAAGA AAATAATCTT GGTATGAGAG
 301 AGTGAGATTG AGCAAAAAAT TTTGAAGAGG TCTTAGCCTT TTATATGCGT TCAAAGTGGA
 361 GGAATTTTGG AAATATCCAT GTATAATGAG ACAAAATCTG CATTTAAAAT GGCATTTCGC
 421 GTCGCCTGCG TCGTGCGAGT GCGCCCCAAC CCTGACGGGT TTGGACTTAC ACCCTCATAC
 481 ACGCGAGGCA GGATTCCAAG TTTAGTCATT CAATCACTCT TAAAGTGAGC TTCAAGCTTA
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 661 GAAGAGAACC GTCGCTCGAA GAGGATGATG ATTCATCCTA TTCATGCACG ACTGTCCAAC
 721 TCCCCACCCA ATCAAATTCC AAATTATGAC ATGAGAAGAA CATCATCCCA CGTGGTCTGT
 781 GCTTCACGCC ACCATGTCCC ACGTGGGCTC CATTTTGGTG GGGCCCTTCC CCACCGCCCA
 841 AGCTGATCCC GGGTTGGCCA TCCCTACTTT TAATTATCAG AGCCACCTCC CCAATCTGCA
 901 AAACGACGGA AATGGAAAAC TATAATTTTC TTTTTTTTCA ACGTACTTAT AAAATATTTT
 961 TCAAAAAAGT ATGAATAAAA TTGTGATATT GCTTGGCCTA AGAGGCCAAT CTTTTGCAAA
1021 TCTCGAAGTC GGGAGGCACA ATAAAAACTT GGAAAGTTTT TTCAAGTGTC TGCTTTATAA
1081 AATTATTGAA ATGCATGTAT TCGTACTTGC CTTATTTATC GACAATTTAA ACATTATTAT
1141 TTCATGAAAA TGTCCTTCCA CCGATTTCAA TGACAAAACC AATAATTACT ACTTTTTATT
1201 TTCAATTATG TCACGGTTCA CATGTTTATT AGGGTTTAGG TTGAGGTTAA AACTTTCGAC
1261 TCTCTATTCG TAACGCTTAA AGATGTAGGG TTTAGGTTGA GGTTAAAACA ATCATGTAAT
1321 GTAAGGATAC CTGAAAAGCT GTCATTAGTG TAAGTGTTTA TTACTAGGGT TGTTTAAATT
1381 CATGTTGATG TCAAGCTTGG ATAACCCATT TTACTAAAAA AATAAATGAA GTCCCAAAGG
1441 GCATTGGGCA TCCTATCAAA GATGGGAAAT TTTTTCAAAA TTTTAACCTA AAAAAGAGGT
1501 GGAAAGTCTT AGTCCAAATA ATCAGCCACA TCAGAATTTG ATTCGTTTCT TTCAAGCAAA
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1741 AACCTTAATT ATTATCACTA GTATTAAATT TCAAAACTTG ATTTGTCCTA AATTTGAAAA
1801 GGGGTCTCCT TCAATTCATA TATGTAGTCA TGAAGATTAT AACTTAGCTG AAAATGGCCT
1861 CCATTATTTG GCTTATTCAA TCAAAAGTTT ACAAAACTAG TGCAAATTTA ATATGATAAT
1921 GTCTACAAGA ACCAAATACG AATTGAGTAA ATTTTTTTGG CTAAAATAAA TTACGAATTG
1981 ATGAATTATC ATTTTAAAAA GTTCTTTTTA ACCATTTCTT TTACTGAATT AAAAAAAGGT
2041 TTTATTAATC ATATATATTA CAAATTACCC ATTAAGTAGC CAAATTACAA ATTTTAATTC
2101 AATGTAGTCA AACACTGATA GTTTAAACAT GACTCTCTTA AGGTAGCCAA AGCCCGGGCT
2161 TAATTAAGGC GCGCCGGCCA AGTCGGCCGC GGCCGCGTTA TCAAGCTTCT GCAGGTCCTG
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2761 TTGTAAGGAA GAATTCGATA TCAAGCTTGA TATCGGAAGT TTCTCTCTTG AGGGAGGTTG
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FIGURE 4

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   1 TGACCGAAGT TAATATGAGG AGTAAAACAC TTGTAGTTGT ACCATTATGC TTATTCACTA
  61 GGCAACAAAT ATATTTTCAG ACCTAGAAAA GCTGCAAATG TTACTGAATA CAAGTATGTC
 121 CTCTTGTGTT TTAGACATTT ATGAACTTTC CTTTATGTAA TTTTCCAGAA TCCTTGTCAG
 181 ATTCTAATCA TTGCTTTATA ATTATAGTTA TACTCATGGA TTTGTAGTTG AGTATGAAAA
 241 TATTTTTTAA TGCATTTTAT GACTTGCCAA TTGATTGACA ACATGCATCA ATCGACCTGC
 301 AGCCACTCGA GTGGAGGCCT CATCTAAGCC CCCATTTGGA CGTGAATGTA GACACGTCGA
 361 AATAAAGATT TCCGAATTAG AATAATTTGT TTATTGCTTT CGCCTATAAA TACGACGGAT
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 601 TACAAAGCTA TTTGCTTATA ACATATGCGA AAAATTTTGT ACTATAATCA GGGGTAAATT
 661 TAGGAGGGGG CTTGTAGGTC TCGCTTCTCT TAAAATGAAA AATTTTCTAT TTAGTTATTT
 721 AAAATTTTAA AAGTAAAATA TAAAAATTTC ATTTAATCCT TTAAAAATTA TAAAGATATA
 781 GACTATTAAA ATGATGAAAT TACAATTTTA TTATCATAAA AATTATAATT TAATTTCGAC
 841 CCCTAACAAA ATTTTCTGAT TTTGCCCCTA ACTGTAATAT TTGTATAAAA ACATTTTCTT
 901 TTTGCATTTA ATGATTTCTT TAATTCAGTC CAAGAAAGAA ATTTATTAAT TGCATATGCG
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1441 AATCTACTTT AAATTAAGTC CTAAAAATAA TATATCATAC CAAAAAAATT CTTAAAATGA
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1561 AATATCATTA TAAATATAAA TCTATTGAGC ATATGTTTTA AACCAAGTAA TGTTGAGTGC
1621 GGTAGTAAAA CTCATTACAC ATTTTAAGTA GAACGTAGTT CGAACCTTGG AGAAG
```

FIGURE 5

SEQUENCE LISTING

<110> Cerny, Eric
Duong, Can
Hart, Jesse
Huber, Scott
Krieb, Rachel
Listello, Jennifer
Martens, Amy
Sammons, Bernard

<120> Cotton Event MON 88913 and Compositions and Methods for Detection Thereof

<130> 11899.0239.00PC00 (MOBT:239P)

<150> 60/447,184
<151> 2003-02-12

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(71) Applicant *(for all designated States except US)*: **SES EUROPE N.V.** [BE/BE]; Industriepark, Soldatenplein Z2, No. 15, B-3300 Tienen (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WEYENS, Guy** [BE/BE]; SES Europe N.V., Soldatenplein Z2, No. 15, B-3300 Tienen (BE). **BARNES, Steve** [GB/BE]; SES Europe N.V., Soldatenplein Z2, No. 15, B-3300 Tienen (BE). **ROSQUE, Inge** [BE/BE]; SES Europe N.V., Soldatenplein Z2, No. 15, B-3300 Tienen (BE).

(74) Agents: **WEITZEL, David** et al.; Brookes Batchellor, 102-108 Clerkenwell Road, London EC1M 5SA (GB).

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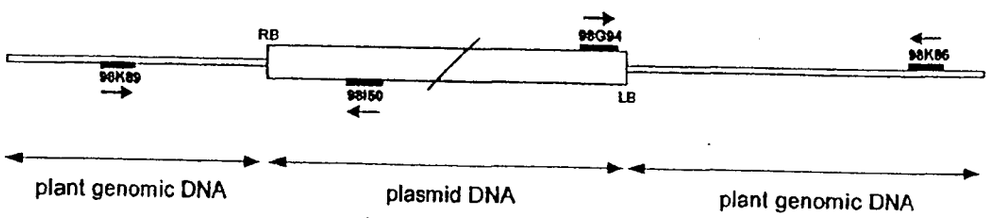
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(54) Title: T227-1 FLANKING SEQUENCE

Transformation event T227-1 with positions of the specific primers



(57) Abstract: A herbicide resistant transformed sugar beet that is detectable by the specific primers developed to match the DNA sequences that flank the left and/or right border region of the inserted transgenic DNA and the method of identifying primer pairs containing plant genomic DNA/plasmid DNA. More specifically the present invention covers a specific glyphosate resistant sugar beet plant having an insertion of the transgenic material identified as the T227-1 event. The present invention additionally covers primer pairs: plant genomic DNA/Plasmid DNA that are herein identified. Additionally, these primer pairs for either the left or the right flanking regions make an event specific test for the T227-1 insert of transgenic material.

WO 02/44407 A2

T227-1 FLANKING SEQUENCES

Invented by:

Guy Weyens having an address at Tienen in Belgium
Steve Barnes having an address at Tienen in Belgium
Inge Rosquin having an address at Tienen in Belgium

FIELD OF THE INVENTION

The field of the invention broadly covers a herbicide resistant transformed sugar beet that is detectable by the specific primers developed to match the DNA sequences that flank the left and/ or right border region of the inserted transgenic DNA and the method of identifying primer pairs containing plant genomic DNA/plasmid DNA. More specifically the present invention covers a specific glyphosate resistant sugar beet plant having an insertion of the transgenic material identified as the T227-1 event. The present invention additionally covers primer pairs: plant genomic DNA/Plasmid DNA that are herein identified. Additionally, these primer pairs for either the left or the right flanking regions make an event specific test for the T227-1 insert of transgenic material. These event specific tests using the primers include a method that employs electrophoresis through gels as the standard method used to separate, identify and purify DNA fragments. The location of the DNA that are separated by size within the gels can be determined by using a dye. This dye permits DNA bands with 1-10 ng of DNA to be detected under ultraviolet light.

BACKGROUND

In the last few years there has been numerous commercial products having glyphosate resistance plants such as soybeans, maize, and rapeseed. At least two different genes have been used to make these commercial products. These products have had a specific event which has been developed through extensive research and testing and then registered with the regulatory authorities in the various countries as genetically modified organisms.

The regulatory requirements in Europe for introduction of plants that have had foreign DNA introduced are most stringent. Although not a specific requirement regulatory offices favor inserted events containing DNA which is necessary for the expression of the introduced trait and very little extra DNA in the insert. On the practical side, the event should have low numbers of copies to avoid problems with gene silencing. Development of these types of insertion events in certain crops such as sugar beets can be difficult. The inserted event should provide the plant with the desired levels of gene expression such as resistance to the application of a herbicide, resistance to insect attack, or production of an oil or sugar, and the like.

Additionally, there is the need to have an insert in sugar beet that is identifiable through tests developed for the inserted event. These test results can be employed to track the GMO in production plants and fields.

There is a need to be able to clearly identify a transgenic plant through its inserted DNA. The need for identifiable transgenic events and the primers and the event specific tests for these primers are increasingly evident.

SUMMARY OF THE INVENTION

Broadly the present invention is to a method of detecting a glyphosate resistant sugar beet comprising the steps of forming primers that flank the genomic and plasmid sequence border in the sugar beet which can uniquely identify the sugar beet, by using a PCR to sequence the unique fragment produced by the primers and detecting the absence or presence of the fragment. The present invention covers a glyphosate resistant sugar beet detectable by this method. Additionally this invention covers a transgenic glyphosate resistant sugar beet comprising an insertion of DNA unique to T227-1 and its progeny. This invention is detectable by either of the pair of primers for the left or right border. The primer set capable of generating a DNA fragment unique to identify T227-1 of 50 base pairs and more preferably approximately 100 base pairs. Wherein the DNA fragment unique to identify T227-1 includes some base pairs related to the sugarbeet genomic DNA and some base pairs related to the inserted plasmid DNA (from pMon17227) in T227-1. A sugar beet that is detectable with a pair of primers in accordance with the present invention that border the left flanking sequence identified as 98G94 and 98K86 generating a 792 bp fragment.

A sugar beet is also detectable with the pair of primers that border the right flanking sequence identified as 98I50 and 98K89 generating a 476 bp fragment. Or the primer set 98K89 - RB12 can also be employed to produce a unique fragment of 246 base pairs.

The invention also includes the pairs of primers comprising DNA which flank at least one of the border regions of the insertion of DNA into the T227-1 Event.
The T 227-1 event is sugarbeet material that is capable of glyphosate resistance when in the plant form and is capable of being detected by at least one of the pair of primers 98G94 and 98K86, 98I50 and 98K89, and/or 98K89 - RB12. The primers should when a positive result is produced identify a DNA fragment of the length associated with that set of primers, negative results should give non DNA fragment or a fragment of a length not associated with the primers. The invention has identified a pair of primers comprising DNA which lies in right border region of insertion of DNA into the T227-1 Event wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

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The invention has also identified a pair of primers comprising DNA which lies in the left border region(Figure 6) of insertion of DNA into the T227-1 Event wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

The primers themselves are generated to identify the T227-1 event by employing a PCR test using a pair of primers. The primers are used in a PCR method to detect the presence or absence of the T227-1 event. A test kit for the T227-1 event can be formed with the primers as components thereof.

The method of detecting the T227-1 event including the steps of selecting sugar beet genomic material for testing; employing at least one of the pair of primers capable of detecting the T227-1 event in association with such selected material; and using a PCR machine to amplify the DNA fragment if it exists; detecting the presence or the absence of the DNA fragment.

The transgenic glyphosate resistant sugar beet of the present invention is characterized by an unique sequence of DNA having at least 80% homology to the right border sequence in Figure 3 or the left border sequence in Figure 6 comprising genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and inserted plasmid DNA.

This invention encompasses a transgenic glyphosate resistant sugar beet wherein at least a 10-20 base pair fragment of said sequence of DNA is capable of detection by a pair of flanking primers wherein one of said primers is developed from the genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and one of said primers being developed from the inserted DNA plasmid.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows the primers used in the flanking PCR test.

FIGURE 2 shows the diagrammatic interpretation of the Southern blot results.

FIGURE 3 shows the plasmid sequence at the right border.

FIGURE 4 shows the sequence of the right border breakpoint.

FIGURE 5 shows the alignment of the right breakpoint region in the transformed sugarbeet T227-1 with the untransformed plant allelic sequence.

FIGURE 6 shows the plasmid sequence at the left border.

FIGURE 7 shows the sequence of the left border breakpoint between the sugar beet genomic material and the inserted plasmid material.

FIGURE 8 shows the alignment of the left breakpoint region and the untransformed plant allelic sequence.

Figure 9 shows the plasmid pMON17227.

Figure 10a shows the sequence of the right border fragment obtained with the 98K89 – RB12 primer set.

Figure 10b shows the sequence of the right border fragment obtained with the 98K89 – 98I50 primer set.

Figure 10c shows the sequence of the left border fragment obtained with the 98G94 – 98K86 primer set.

DETAILED DESCRIPTION OF THE INVENTION

The selection of a specific event in sugar beets (*Beta vulgaris)* requires the production of numerous transformants. These transformants must be screened in most instances to eliminate those transformants that do not conform fairly closely with the genotype of the original germplasm employed. These transformants must be tested for the efficiency of the inserted event. On a molecular basis these transformants must be tested for copy number to mostly to select for low copy number which may avoid gene silencing. Additionally, the transformants are tested and developed and screened based on the positional effect the location in the genome the inserted event has on the gene activity. Different insertion sites in the genome results in different overall results. For example, if the insertion is located in an area with high transcription there may be many copies of the protein produced; if the insertion is located in an area with low transcription, the protein may not be produced at sufficient levels for the desired gene effect. Thus, the selection of an insertion in recalcitrant plant such as sugar beet involves numerous levels of investigative work and research. This type of research and development differs from plant to plant, from gene to gene, and according to the desired results.

The present invention broadly encompasses a sugar beet which is resistant to glyphosate at levels that would normally result in the death of the sugar beet plant. The resistance has been introduced by the *Agrobacterium* mediated transformation of the sugar beet with the pMON17227 plasmid. This plasmid is shown in Figure 9 and it contains the FMV 35S promoter. The 35 S promoter is from a modified figwort mosaic virus. It also contains the chloroplast transit peptide from the EPSPS gene of Arabidopsis. And the CP4 synthetic gene optimized from the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. Strain

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CP4. This plasmid also contains the terminator from pea. The terminator is from the pea gene RBCSE9. The E9 3'is the pea *rbcS* (small subunit ribulose bisphosphate carboxylase oxygenase) E9 gene. The plasmid also includes the left and right T-DNA border sequences from *Agrobacterium*.

Numerous transformants were produced by the *Agrobacterium* mediated transformation of the sugar beet. The plantlets that survived the initial transformation stages were then screened in a green house for herbicide resistance. Levels of differing doses of herbicide were applied to the plant to determine which transformants were sensitive and which were resistant. The sensitive transformants were destroyed by the herbicide application and the resistant plants were subjected to further analysis based on comparison to the genome of the original germplasm employed in the transformation process. The transformants that were not elliminated in this screening were further selected from in further green house experiments. The resulting selected transgenic plants were analysed on a different criteria including on the molecular level.

This analysis led to the selection of a rare event insertion designated T227-1. The transformation method used to generate T227-1 integrated the vector into the genome by breakage of the circular vector at a position that was relative to the left and right border sequences. In the *Agrobacterium* system, the left and right border sequences are specifically recognised by vir genes (virD1 and virD2). These proteins produce nicks in the border sequences and the fragment is transferred from the bacterial cell into the plant cell as a single stranded T-DNA molecule, not as a circular vector.

To characterise T227-1 fully, as well as providing information that could serve as a basis for event-specific PCR tests, the nucleotide sequence of the breakpoints between inserted vector and plant genomic DNA was determined.

FLANKING SEQUENCES – T227-1

Genomic sequences flanking the T227-1 insert were isolated by linker PCR. Genomic DNA was digested with restriction endonucleases and linkers attached to the ends of the fragments obtained. The fragments containing the T227-1 sequences were preferentially amplified by PCR using the linker sequence and pMON17227-specific sequences as primers. The PCR products were sequenced directly, and the sequences used to design further primers, which were used to amplify plant genomic DNA from T227-1 and from untransformed plants.

Genomic plant DNA fragments were isolated for both left and right borders. These linker PCR products were sequenced and these sequences were aligned with (a) pMON17227 and (b) the fragments generated by the plant genomic primers using plant DNAs (containing the transformed and untransformed "alleles") as templates.

Although the sequence contains some ambiguities, these are essentially consistent with the sequences being identical.

RIGHT BREAKPOINT

The present invention has its sugar beet genetic makeup at the insert sites in the right and left border regions identified and defined. In FIGURE 3 the plasmid sequence at the right border is shown. The inserted DNA sequence was from pMON17227. The top portion of the drawing shows the sequence of the right border of the insert, the sugar beet genome and the beginning of the inserted foreign DNA. The Sub 105, the RB7 and the RB11 are small primer regions. The various combinations of primer regions can be employed singly or in pairs. The break at 7965 base pairs (indicated by the arrow) shows where the insertion has occurred. The DNA before this break is genomic sugar beet and after the break is the sequence from the plasmid.

FIGURE 4 shows the sequence of the right border breakpoint and where the primers lie on this sequence. In the fourth row of sequence data the bolded sequence indicates the DNA sequence of the 98K89 primer. The bolded sequence in the sixth line of sequence indicates the restriction site of *Bgl*II. The sequence that is in the greyed in area is the beginning of the integrated plasmid material from pMON17227. Within the greyed area are two separate bolded portions of sequence. The first bolded sequence portion is the *Hind*III restriction site. The second bolded sequence portion is the RB12 primer sequence. The pair of primers, when employed, allow the PCR fragment of DNA that is also unique to this event to be generated.

FIGURE 5 shows the alignment of the right breakpoint region in the transformed sugar beet T227-1 with the untransformed plant allelic sequence. The sequence matches up so that it shows that the gene in the untransformed plant and the T227-1 event match. Certain base pairs could not be identified as ACTG and the following classification was used as listed in Table 1.

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Table 1

S=G or C

R=A or G

W=A or T

K=G or T

M=A or C

Y=C or T

N=any base

B=C, G or T

There is a 17 base pair fragment in the last line of the sequence before the start of the plasmid sequence that indicates that there are 17 base pairs in the T227-1 transformed sugar beet that differs from the untransformed. After this 17 base pair fragment, which is in italics, the plasmid sequence data is found.

The alignments of sequence data at the right side of the integration site are shown in Figure 5. The nucleotides common to both the transformant (T227-1) and the plasmid (pMON17227) are indicated by an asterisk (*) *below* the aligned sequence.

Between the vector breakpoint and the beginning of homology with beet genomic sequences, a 17bp fragment is found which is not present in the untransformed allelic sequences studied in this germplasm. No perfect homology could be found between this short unknown DNA sequence and the plasmid sequence.

These results are entirely consistent with the Southern and PCR analyses of T227-1.

LEFT BREAKPOINT

FIGURE 6 shows the plasmid sequence at the left border. The inserted DNA sequence was from pMON17227. The top portion of the drawing shows the sequence of the left border of the insert and the associated sugar beet genome. The base pair 2336 is a portion of the left border of the inserted DNA material. The Sub 103, Sub 104, LB8 and the LB9 are small primer regions. The break at 2686 base pairs (indicated by the arrow) shows where the insert border has occurred. The DNA before this break is the sequence from the plasmid and after the break is genomic sugar beet.

FIGURE 7 shows the sequence of the left border breakpoint between the sugar beet genomic material and the inserted plasmid material. The greyed in region is the integrated plasmid sequence. At the end of this sequence is the bolded primer LB8. In the next line is the bolded sequence that indicates the restriction site for *Bgl*II. The primer that is located in the sugar beet genomic material is identified in bold in the figure at the bottom.

FIGURE 8 shows the alignment of the left breakpoint region and the untransformed plant allelic sequence. The T227-1 transformed event starts with the plasmid sequence. Then there is a region of sparse matching between the untransformed and the T227-1, a fragment of 49 base pairs. Thereafter there is a close correlation between the untransformed sugar beet and the transformed sugar beet called event T227-1.

The alignments of sequences from the left side of the integration site and its known component elements are shown in Figure 8.

At the 3' end of the sequence there is clear homology to the pMON17227 vector though there is a 49 base pair fragment that sparsely aligns with the untransformed beet "allele", or to pMON17227.

On the basis of the sequence shared by the transformant and the plasmid it is clear that a 4 base pair section of the left T-DNA border was incorporated into the Event T227-1 during or following the transformation event. No right T-DNA border and very little of the left T-DNA border portions, were integrated into the transgenic sugar beet event entitled T227-1.

Beyond this 4 base pair section of the left T-DNA border region from *Agrobacterium* the plasmid sequence has been entirely integrated, excluding the right border section of the *Agrobacterium* region, after which point recombination has taken place to insert the construct into the beet genome.

These results are fully consistent with the Southern hybridisation results.

USE OF FLANKING PRIMERS TO ANALYSE T227-1 AND UNTRANSFORMED SUGAR BEET

Specific primers were developed from the left and right flanking sequences, and used in PCR reactions with each other or individually with primers from within the insert. Primer locations in the inserted DNA and in the sequence flanking the insert are shown in Figures 1, 3-7 and Table 1.

PCR reactions between a flanking primer and an insert primer always gave the size expected from the sequence information, and have never given any product in untransformed beet or transformants other than T227-1. Thus these sequences are used as an event-specific test whereby the T227-1 event is identified. This identification process of the insertion of event T227-1 which is one copy and has little extraneous DNA and is highly efficacious at resisting the effects of glyphosate on the plant, is possible in the original germplasm and in progeny developed by any manner of breeding or selfing. The material from the progeny including pollen and seeds and the like can be tested for the presence of this insert in the DNA.

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GENOMIC FLANKING SEQUENCES

The plasmid shown in Figure 9 contained the LB (left border) RB (the right border), the FMV 35S promoter (from a modified figwort mosaic virus), and the main gene of interest to give glyphosate resistance the CP4 syn: the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium sp.* Strain CP4. The plasmid also contains a chloroplast transit peptide and a terminator from pea. The circular vector pMON17227 opens at both left and right borders when inserted into the plant genome to form the transformed Event. The selected transformation event was the T227-1 Event. This transformed beet T227-1 and its progeny show excellent levels of glyphosate tolerance.

To characterize T227-1 fully, providing information that serves as a basis for event-specific PCR tests, the nucleotide sequences of the breakpoints between inserted vector and plant genomic DNA were determined.

Genomic sequences flanking the T227-1 insert were isolated by linker PCR. Genomic DNA was digested with restriction endonucleases and linkers attached to the ends of the fragments obtained. The fragments containing the T227-1 sequences were preferentially amplified by PCR using the linker sequence and pMON17227-specific sequences as primers. The PCR products were sequenced directly, and the sequences used to design further primers; these were then used to amplify plant genomic DNA from the T227-1 transformant for both left and right borders (flanking sequences PCR) and from an untransformed plant.

FIGURE 1 shows the primers used in the flanking PCR test. The first pair of primers is in the right border region of the inserted material. 98k89 is a primer that is located within the sugar beet plant genome. 98I50 is a primer that is located in the inserted plasmid DNA sequence. In combination these primers can be employed to produce a unique piece of DNA through a PCR test. This unique DNA specifically identifies this T227-1 transformation event. The sugar beet identified by this PCR flanking sequence is only the T227-1 event or its progeny.

The second pair of primers is in the left border region of the inserted material. 98K86 is a primer that is located within the sugar beet plant genome. 98G94 is a primer that is located in the inserted plasmid DNA sequence. In combination these primers can be employed to produce a unique piece of DNA through a PCR test. These event specific tests using the primers include a method that employs electrophoresis through gels as the standard method used to separate, identify and purify DNA fragments. The location of the DNA that is separated by size within the gels can be determined by using a dye. This dye permits DNA bands with 1-10 ng of DNA to be detected under ultraviolet light. This unique DNA specifically identifies this T227-1 transformation event. The sugar beet identified by this PCR flanking sequence is only the T227-1 event or its progeny. Test kits comprising at least one set of primers wherein one such primer is located within the sugar beet plant genome proximate the insert and the other such primer is located within the inserted plasmid DNA sequence of T227-1, wherein an unique piece of DNA is capable

of being identified wherein the material is identified as being GMO positive or GMO negative Additionally the material can be more specifically identified as being T227-1 or its progeny.

Primers 98K89 and 98K86 for the T227-1 event or its progeny are situated in the regions flanking the inserted sequences, while 98I50 and 98G94 lie inside the DNA from the vector. The resulting PCR products are described in Table 2.

Table 2: Specific primers inside and outside the integrated vector sequence.

	Vector specific primer (position)	Flanking DNA primer	PCR product
Left border	98G94 (2503bp)	98K86	792 bp
Right border	98I50 (8232bp)	98K89	476 bp

PCR with these primers was performed on a 9600 thermocycler from PE-Biosystems. In each 50 µl reaction, 50 ng of plant DNA was incubated with 120 ng of each primer and 1U of Amplitaq DNA Polymerase in GeneAmp PCR buffer II containing 10mM Tris-HCl pH8.3 and 50mM KCl complemented with 1.5mM $MgCl_2$ and 0.2mM dNTP. One PCR reaction consisted of a hot start of 3 min. at 94°C followed by 35 cycles (one cycle: 30 sec. at 94°C, 1 min. at 57°C and 1 min. at 72°C). The PCR products were separated on a 1.5% agarose gel.

This amplification clearly demonstrates that the left and right flanking sequences are normally contiguous in sugar beet, and have not suffered major reorganisation as a result of the transformation.

FLANKING SEQUENCES – PROGENY OF T227-1.

By sequencing the flanking regions we developed specific primers from the left and right flanking sequences, and used these in PCR reactions with each other or individually with primers from within the insert. Such a PCR assay can be used to identify the T227-1 transformation event and its progeny uniquely.

Primer pairs (plant genomic DNA / plasmid DNA) for both the left and the right region were tested on T227-1, its progeny and the non-transformed lines. Both primer combinations gave positive results for all the glyphosate resistant plants (all generations). All these PCR products were identical to those obtained with transformation event T227-1. The fragment lengths of a few of the combinations are listed in Figures 10a-10c. Other primer pairs can give different fragments. The glyphosate sensitive plants and the non-transformed line, however, showed no amplification product at all.

The T227-1 event and its progeny were tested for this unique DNA by using Southern blots. Figure 2 shows the diagrammatic interpretation of the Southern blot results. The four boxes on top of the figure depict the plasmid sequence DNA that was incorporated into the transformed sugar beet T227-1. The components of the plasmid differ somewhat from the inserted material. The sugar beet was transformed through the use of *Agrobacterium*. Therefore, the plasmid contained both the right and left T-DNA border regions. The right T-DNA border sequence was not transformed into T227-1. The left T-DNA border sequence was not transformed except for a small 4 base pair section that is integrated between the sugar beet plant genome and the E9 3' terminator sequence.

The inserted material is identified as FMV, CTP 2, CP4 syn, E9 3', LB. FVM is a 35S promoter from a modified figwort mosaic virus. CP4 syn is the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium sp.* Strain CP4. CTP2 is the chloroplast transit peptide from Arabidoposis. E9 3' is the terminator. LB is the small portion of the left border sequence from *Agrobacterium*.

The vertical arrows depict the restriction sites located in the genome and the inserted material. The horizontal lines below the top two boxes depict the approximate number of base pairs that are in a fragment that is produced by employing the restriction enzyme indicated on the top box. The second box depicts the transformed sugar beets genetic makeup at the insert site.

The beet genomic sequences flanking this insert have been sequenced, and employed to design specific PCR tests for the T227-1 insertion. Flanking primers may be used for amplification of untransformed sugar beet DNA, indicating that the transformation did not induce any major rearrangements in the beet genome. This is confirmed by sequence analysis, which shows that the sequences on either side of the insert are essentially collinear with those of untransformed "alleles" from the same locus. Thus the present invention includes a method of using primers that identify T227-1 in a PCR method of detecting DNA. The invention also covers each of the two pair of primers that flank the right and left borders. The invention further covers the sugar beet having the DNA that is detectable by the primers.

We Claim:

1. The transgenic glyphosate resistant sugar beet comprising an insertion of DNA unique to T227-1 and its progeny detectable by either of the pair of primers for the left or right border.

2. A sugar beet according to claim one that is detectable with the pair of primers identified as 98G94 and 98K86.

3. A sugar beet according to claim one that is detectable with the pair of primers identified as 98I50 and 98K89.

4. A pair of pimers comprising DNA which flank one of the border regions of the insertion of DNA into the T227-1 Event.

5. A pair of primers comprising DNA which lies in right border region of insertion of DNA into the T227-1 Event.

6. A pair of primers according to claim 5 wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

7. A pair of primers comprising DNA which lies in the left border region of insertion of DNA into the T227-1 Event.

8. A pair of primers according to claim 7 wherein one primer lies in the plant genomic material and the other primer is in the inserted plasmid material.

9. A PCR test using a pair of primers according to claim 4.

10. Using the primers according to claim 4 in a PCR method to detect the presence or absence of the T227-1 event.

11. The method of detecting the T227-1 event including the steps of:
    1) Selecting sugar beet genomic material;
    2) Employing the pair of primers capable of detecting the T227-1 event in association with such selected material;
    3) Using a PCR machine to amplify the DNA fragment if it exists;
    4) Detecting the presence or the absence of the DNA fragment.

12. A sugar beet according to claim one that is detectable with the pair of primers identified as 98K89-RB12.

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13. A sugar beet according to claim one that is detectable with the pair of primers that produce a fragment selected from the lengths consisting of 246 base pairs, 476 base pairs or 792 base pairs.

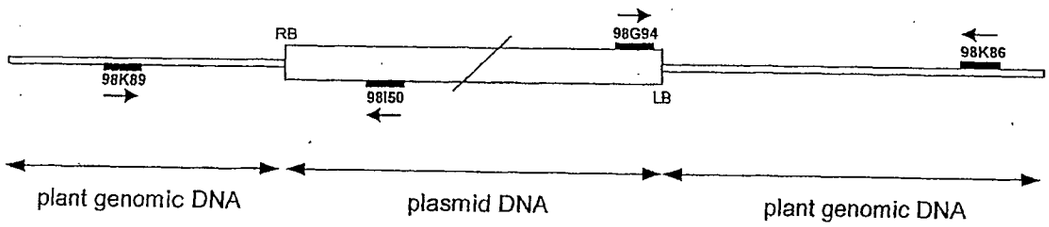
14. The method of detecting a glyphosate resistant sugar beet characterized by the T227-1 event with a test comprising the steps of:

1) Selecting sugar beet genomic material for testing;
2) Forming a pair of primers flanking the border sequence of Figure 3 or Figure 6 wherein said primers are capable of detecting the glyphosate resistant sugar;
3) Using a PCR machine to amplify a unique DNA fragment produced through the use of the primers, if such fragment can be produced; and
4) Detecting the presence or the absence of the DNA fragment.

15. The transgenic glyphosate resistant sugar beet characterized by an unique sequence of DNA having at least 80% homology to the right border sequence in Figure 3 or the left border sequence in Figure 6 comprising genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and inserted plasmid DNA.

16. The transgenic glyphosate resistant sugar beet according to claim 15 wherein at least a 20 base pair fragment of said sequence of DNA is capable of detection by a pair of flanking primers wherein one of said primers is developed from the genomic sugar beet DNA proximate the breakpoint of the insertion of the DNA into the genome and one of said primers being developed from the inserted DNA plasmid.

FIGURE 1: Transformation event T227-1 with positions of the specific primers



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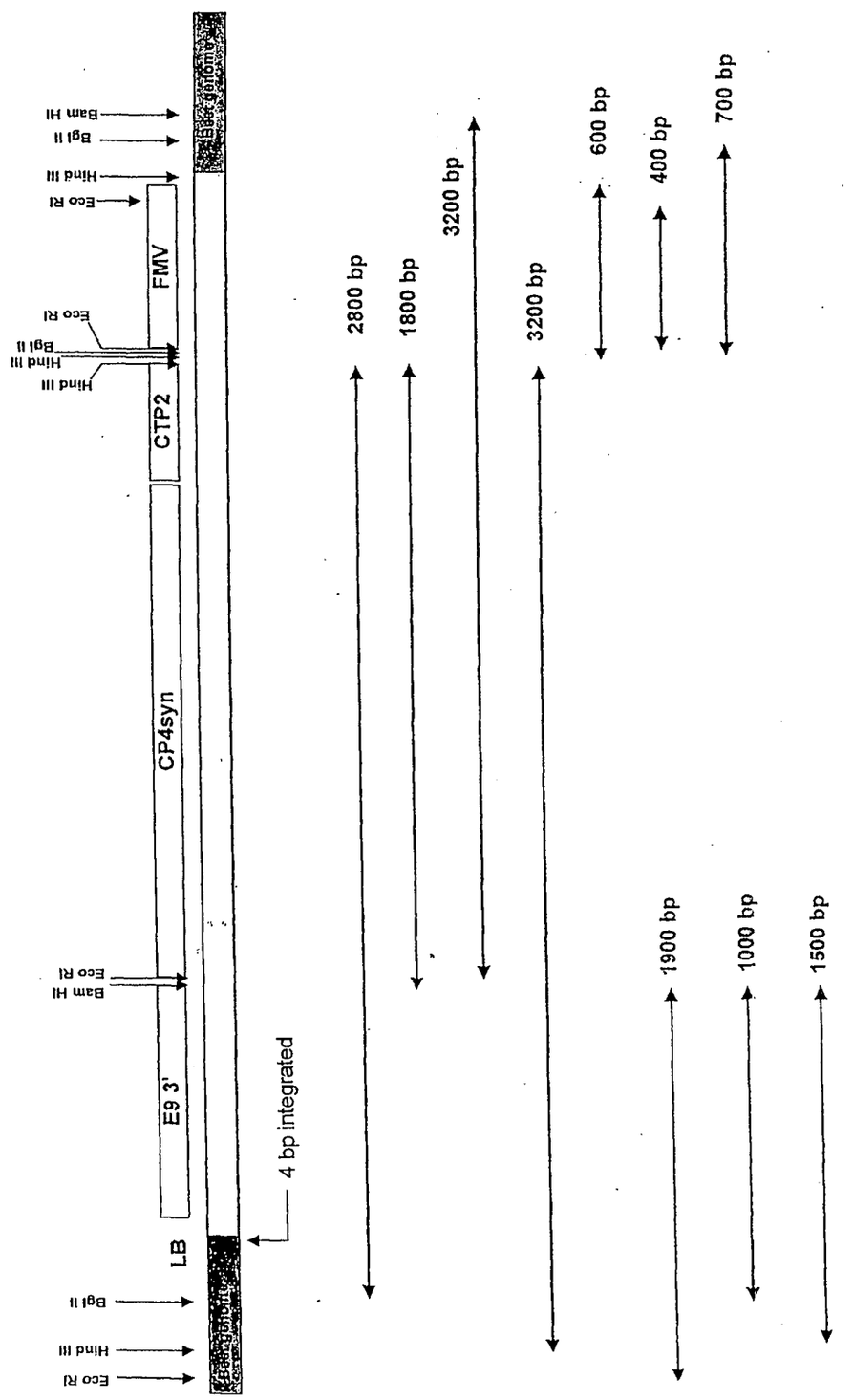


FIGURE 2
Diagrammatic interpretation of the Southern blot results.

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FIGURE 3

Plasmid sequence at the right border.

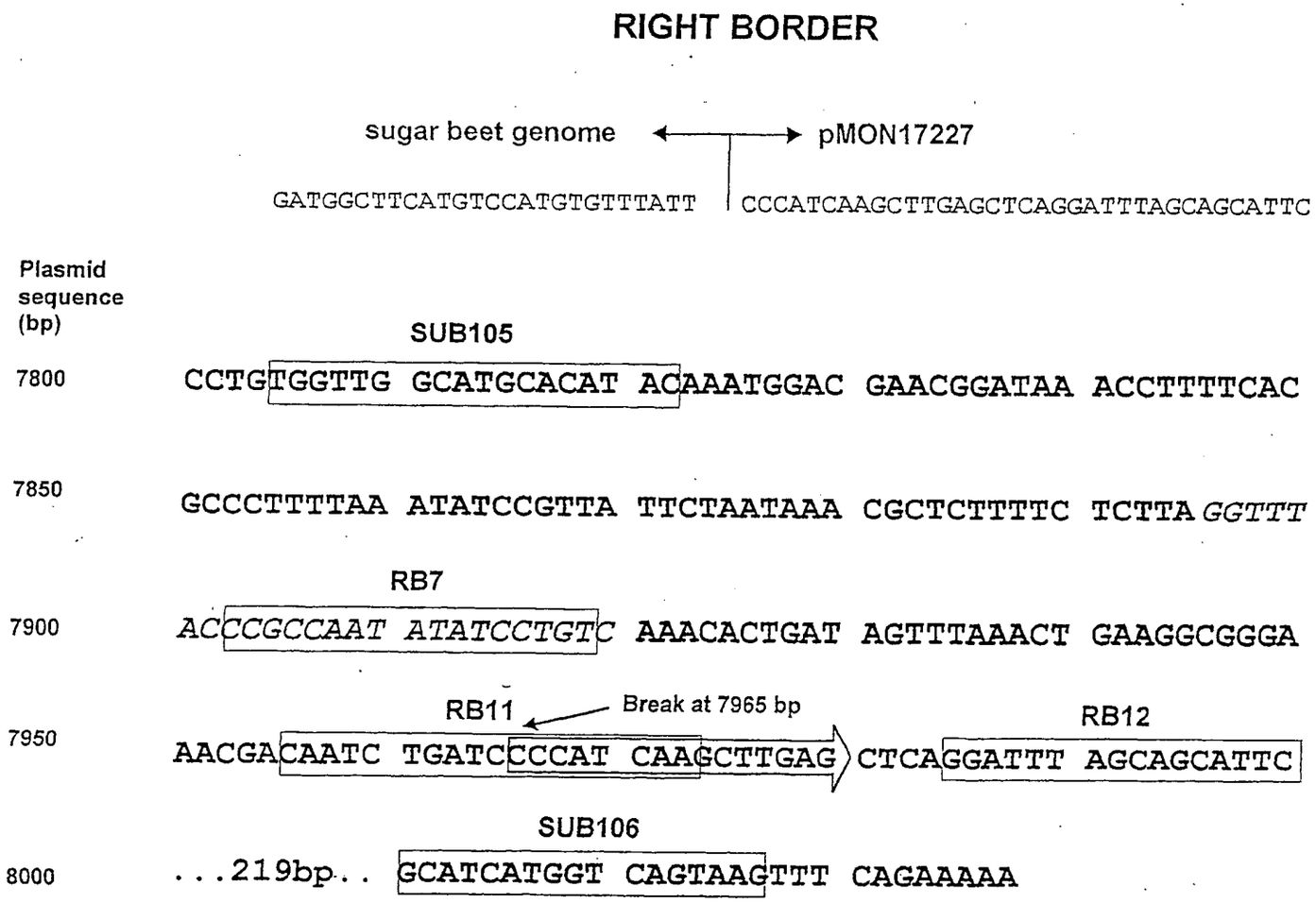
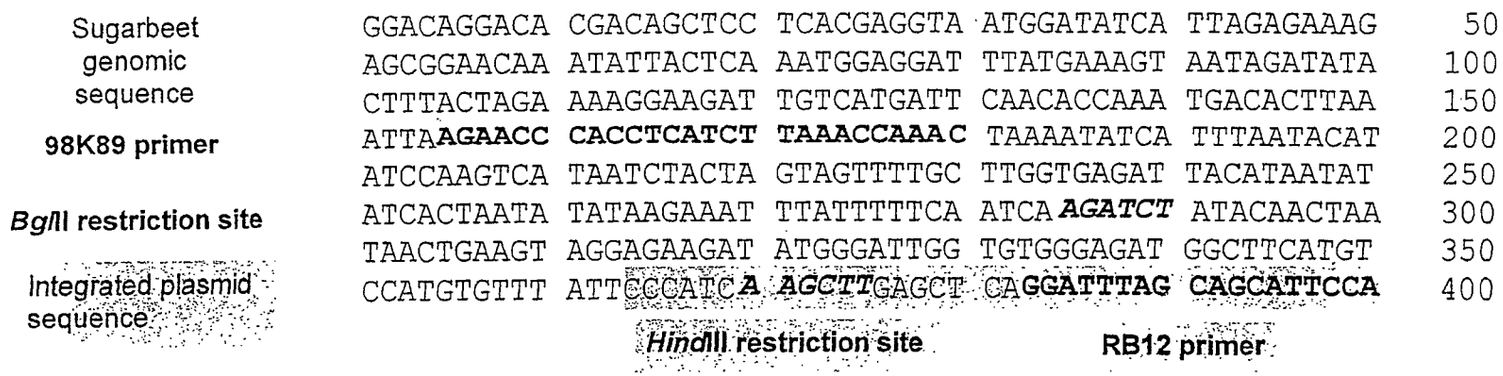


FIGURE 4

Sequence of the right border breakpoint.



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FIGURE 5

Alignment of the right breakpoint region and the untransformed plant allelic sequence.

```
UNTRANSF                                    ATTTAAATRTC
                                            * * *******

T227-1 right    AATTAAGAACCCACCTCATCTTAAACCAAACTAAAATATC

UNTRANSF              WKTTGMCACATATCCAAGTCATAATCTACKASYAGTTTKG
                      **** * **********************************

T227-1 right    ATTTAATACATATCCAAGTCATAATCTACTAGTAGTTTTG

UNTRANSF              CTTGGTGAGATTACATAATAKATCACTAATATATAASAAA
                      ****************************************

T227-1 right    CTTGGTGAGATTACATAATATATCACTAATATATAAGAAA

UNTRANSF              TTTATTTWTCAATCAAGATCTATACAACTAATWMCTGAAG
                      ****************************************

T227-1 right    TTTATTTTTCAATCAAGATCTATACAACTAATAACTGAAG

UNTRANSF              TAGGAGAAGATATGGGATTGGTGTGGGASATGGATTCCCC
                      ********************************** ***

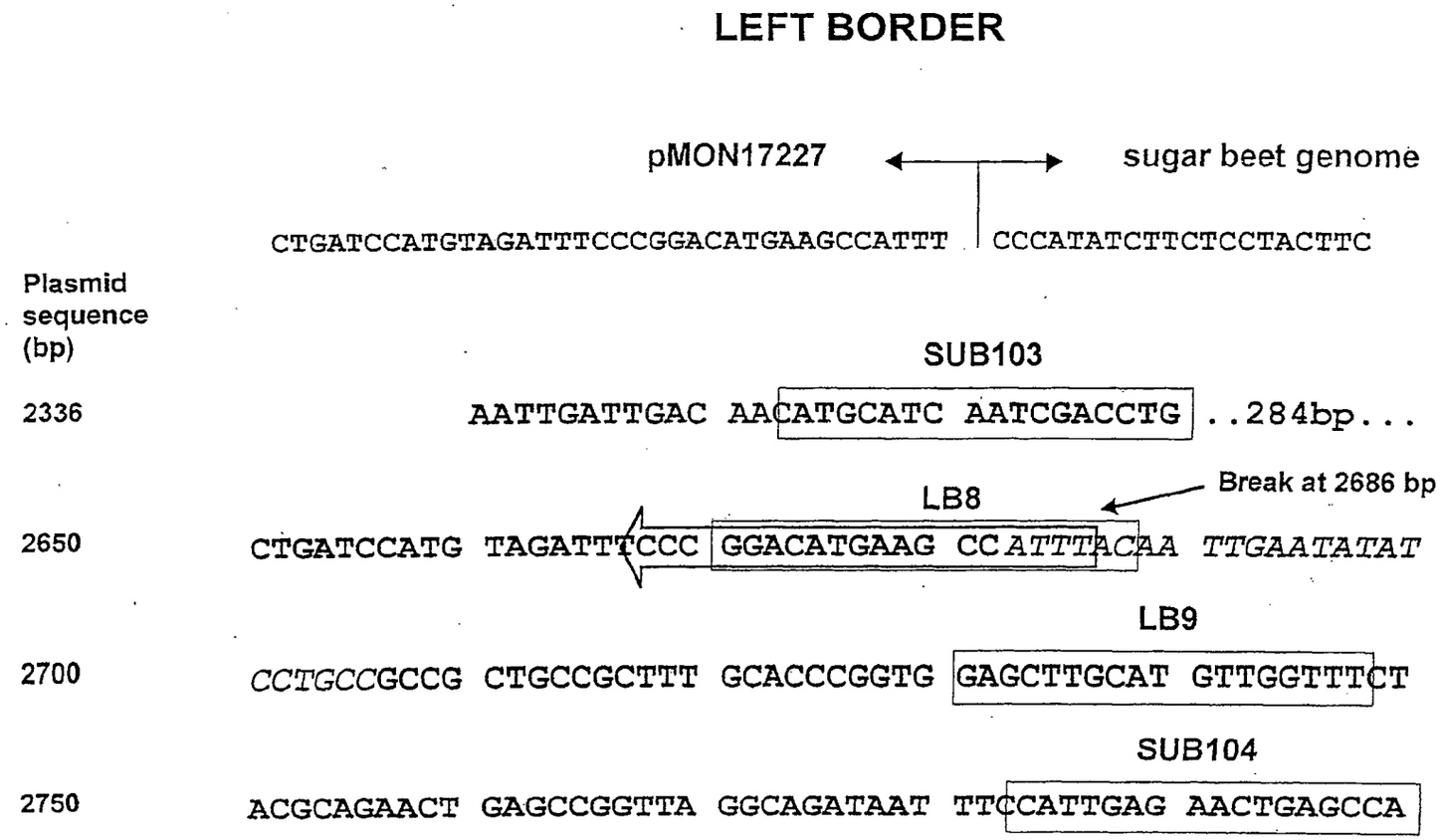
T227-1 right    TAGGAGAAGATATGGGATTGGTGTGGGAGATGGCTTCATG

UNTRANSF              ATATAAAGTAAAGAGAGTCAA

T227-1 right    TCCATGTGTTTATTCCCATCAAGCTTGAGCTCAGGATTTA
                17bp fragment         plasmid sequence
```

FIGURE 6

Plasmid sequence at the left border.



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FIGURE 7

Sequence of the left border breakpoint.

Integrated plasmid sequence **LB8 primer**	AATGTNCTTT CATTTTATAA NAACGCTGCG GACATCTACA TTTTTGAATT	50
	GANAAAAAAT TGGTAATTAC TCTTTCTTTT TCTCCATATN GACCATCATA	100
	CTCATTGNTG ATCCATGTAG ATTTCCC**GGA CATGANGCCA TTT**CCCATAT	150
***Bgl*II restriction site**	CTTCTCCTAC TTCNAGTCNA TTAGTTGTAT ***AGATCT***TGAT TGAAAAATAA	200
	ATATTTGTCC CAACTCTCTT TTATTCCCTG TGTCCATGTC TGAACAACTT	250
	TCGAATTTTC TTCCTAATAA TCTCGCGATA ACTTGCATGG TTTGGAACAT	300
	GCAATGAGCG AGAAATANAA ATTTTATTTC TGCTTTGAAA GCAATTGTTA	350
Sugarbeet *genomic* sequence	GAATGCATCG TCCTACTGTG ATTGCATGAG TGGAAACACA TATGGGAGGA	400
	AATCAAGCTA TGTCTATTGC ATCTGCTCTG GGGTACTCTG GTCATACTCG	450
	TGTCGATGCC ATGGGTTTTT TAGGGGGAAT TTTGATTTAT TGGAAACCAG	500
	AATTGGTTAC CATAGAACCT ATCATTAGAC ATGCATGATC AACATATAAC	550
	CATGGAAATA AAAAGGGTAG GGGCTATTCT TTGGTATTTC TCAGCGGTTT	600
	ATGCGAGTCC CGACCCTACA AAACGCCAAG TTCTTTGGCA AGAATTAAGA	650
***Hind*III restriction site**	AATTTCGCTC GAACTCATAA TC***AAGCTT***GG CTCATAGCAG GAGATTTTAA	700
98K86 primer	TGATACCAGA TATTCCTATG AAAGGAATA**C TGCTTGTTCG GAAACTCAAC**	750
	GTTGTCTCTT AGTTTCAATG ATTGGGTNNN TGACATGGAT TAATGAA	797

FIGURE 8

Alignment of the left breakpoint region and the untransformed plant allelic sequence.

```
UNTRANSF          ATATGGGAT-TGGTGTGGGASATGGATTCCCCATAT
                  *** ** * * * * ** ** **
T227-1 left       AAGCCATTTCCCATATCTTCTCCTACTTCNAGTCN---AT
      plasmid sequence                    49bp fragment

UNTRANSF          AAAGTAAAGAGAGTCAACAAGAAGAWATAAAATATTTGTCC
                  * * * *** * * * ******* *********
T227-1 left       TAGTTGTATAGA-TCTTGATTGAAAAATAAA-TATTTGTCC

UNTRANSF          CAACTCTCTTTTATYCC-TGTGTCCATGTCTGAACAACTYT
                  ********** ********** **********
T227-1 left       CAACTCTCTTTTATTCCCTGTGTCCATGTCTGAACAACTTT

UNTRANSF          CGAATTTTCTTCCTAATAATCTCGCGATAACTTGCATGGTT
                  ****************************************
T227-1 left       CGAATTTTCTTCCTAATAATCTCGCGATAACTTGCATGGTT

UNTRANSF          TGGAACATGCAATGAGCGAGAAATAGAAATTTTATTTCTGC
                  ****************************************
T227-1 left       TGGAACATGCAATGAGCGAGAAATANAAATTTTATTTCTGC

UNTRANSF          TTTGAAAGCAATTGTTAGAATGCATCGTCSTACWGTGATTG
                  ****************************************
T227-1 left       TTTGAAAGCAATTGTTAGAATGCATCGTCCTACTGTGATTG

UNTRANSF          CATGAGTGGAAACACATATGGGAGGAAATCAASCTATGTCT
                  ****************************************
T227-1 left       CATGAGTGGAAACACATATGGGAGGAAATCAAGCTATGTCT

UNTRANSF          ATTGCATCTGCTCTGGGGTACTCTGGTCATACTCGTGTCGA
                  ****************************************
T227-1 left       ATTGCATCTGCTCTGGGGTACTCTGGTCATACTCGTGTCGA

UNTRANSF          TGCCATGGGTTTTTTAGGGGGAATTTTGATWTATTGGAAAC
                  ****************************************
T227-1 left       TGCCATGGGTTTTTTAGGGGGAATTTTGATTTATTGGAAAC
```

```
UNTRANSF        CAGAATTGGKWRCCATAGAACCTATCATTAGACATGCATGA
                ****************************************
T227-1 left     CAGAATTGGTTACCATAGAACCTATCATTAGACATGCATGA

UNTRANSF        TCAACATATAACCATGGAAATAAAAAGGGTWGGGGCTATTC
                ****************************************
T227-1 left     TCAACATATAACCATGGAAATAAAAAGGGTAGGGGCTATTC

UNTRANSF        CTTTGGTATTTCTCAGCGGTTTATGCGAGTCCSGACCCTAC
                ****************************************
T227-1 left     -TTTGGTATTTCTCAGCGGTTTATGCGAGTCCCGACCCTAC

UNTRANSF        AWAACGCCAAGTTACTTTGGCAAGAATTAAGAAATTTCGCT
                ************ ************************
T227-1 left     AAAACGCCAAGTT-CTTTGGCAAGAATTAAGAAATTTCGCT

UNTRANSF        CGAACTCATWMTCAMGCTKSGCTCATRGCMSGAGAWTTTAA
                ****************************************
T227-1 left     CGAACTCATAATCAAGCTTGGCTCATAGCAGGAGATTTTAA

UNTRANSF        TGWK-CCARATKBCCTATGAAAGGAAA
                **** ******** *    **  **
T227-1 left     TGATACCAGATATTCCTATGAAAGGAATACTGCTTGTTCGG
```


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FIGURE 9 Restriction sites of the enzymes on pMON17227.

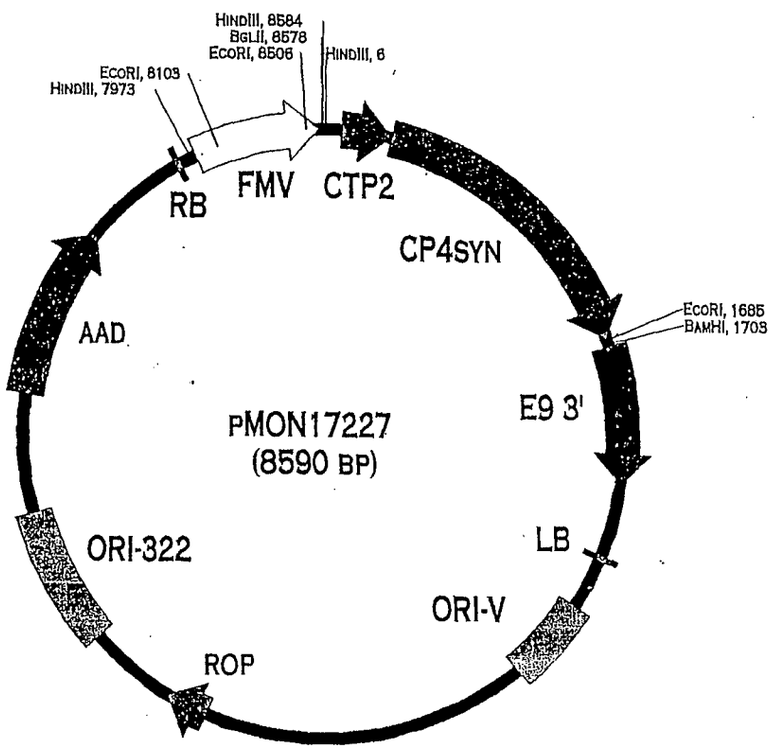


Figure 10a

Sequence of the right border fragment obtained with the 98K89 – RB12 primer set.

98K89 primer

AGAACCCACC TCATCTTAAA CCAAACTAAA ATATCATTTA ATACATATCC 50
AAGTCATAAT CTACTAGTAG TTTTGCTTGG TGAGATTACA TAATATATCA 100
CTAATATATA AGAAATTTAT TTTTCAATCA AGATCTATAC AACTAATAAC 150
TGAAGTAGGA GAAGATATGG GATTGGTGTG GGAGATGGCT TCATGTCCAT 200
GTGTTTATTC CCATCAAGCT TGAGCTCA**GG ATTTAGCAGC ATTCCA** 246

RB12 primer

Figure 10b

Sequence of the right border fragment obtained with the 98K89 – 98I50 primer set.

98K89 primer

AGAACCCACC TCATCTTAAA CCAAACTAAA ATATCATTTA ATACATATCC 50
AAGTCATAAT CTACTAGTAG TTTTGCTTGG TGAGATTACA TAATATATCA 100
CTAATATATA AGAAATTTAT TTTTCAATCA AGATCTATAC AACTAATAAC 150
TGAAGTAGGA GAAGATATGG GATTGGTGTG GGAGATGGCT TCATGTCCAT 200
GTGTTTATTC CCATCAAGCT TGAGCTCAGG ATTTAGCAGC ATTCCAGATT 250
GGGTTCAATC AACAAGGTAC GAGCCATATC ACTTTATTCA AATTGGTATC 300
GCCAAAACCA AGAAGGAACT CCCATCCTCA AAGGTTTGTA AGGAAGAATT 350
CTCAGTCCAA AGCCTCAACA AGGTCAGGGT ACAGAGTCTC CAAACCATTA 400
GCCAAAAGCT ACAGGAGATC AATGAAGAAT CTTCAATCAA AGTAAACTAC 450
TGTT**CCAGCA CATGCATCAT GGTCAG** 476

98I50 primer

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Figure 10c

Sequence of the left border fragment obtained with the 98G94 – 98K86 primer set.

98G94 primer

CGCCTATAAA TACGACGGAT CGTAATTTGT CGTTTTATCA AAATGTACTT 50
TCATTTTATA ATAACGCTGC GGACATCTAC ATTTTTGAAT TGAAAAAAAA 100
TTGGTAATTA CTCTTTCTTT TTCTCCATAT TGACCATCAT ACTCATTGCT 150
GATCCATGTA GATTTCCCGG ACATGAAGCC ATTTCCCATA TCTTCTCCTA 200
CTTCNAGTCN ATTAGTTGTA TAGATCTTGA TTGAAAAATA AATATTTGTC 250
CCAACTCTCT TTTATTCCCT GTGTCCATGT CTGAACAACT TTCGAATTTT 300
CTTCCTAATA ATCTCGCGAT AACTTGCATG GTTTGGAACA TGCAATGAGC 350
GAGAAATANA AATTTTATTT CTGCTTTGAA AGCAATTGTT AGAATGCATC 400
GTCCTACGTG ATTGCATGAG TGGAAACACA TATGGGAGGA AATCAAGCTA 450
TGTCTATTGC ATCTGCTCTG GGGTACTCTG GTCATACTCG TGTCGATGCC 500
ATGGGTTTTT TAGGGGGAAT TTTGATTTAT TGGAAACAGA ATTGGTTACC 550
ATAGAACCTA TCATTAGACA TGCATGATCA ACATATAACC ATGGAAATAA 600
AAAGGGTAGG GGCTATTCTT TGGTATTTCT CAGCGGTTTA TGCGAGTCCC 650
GACCCTACAA AACGCCAAGT TCTTTGGCAA GAATTAAGAA ATTTCGCTCG 700
AACTCATAAT CAAGCTTGGC TCATAGCAGG AGATTTTAAT GATACCAGAT 750
ATTCCTATGA AAGGAATA**CT GCTTGTTCGG AAACTCAACG TT** 792

98K86 primer

US 20030199681A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2003/0199681 A1

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(54) NOVEL PLANT EXPRESSION CONSTRUCTS

(75) Inventors: **Karen L. Fincher**, Pacific, MO (US); **Stanislaw Flasinski**, Chesterfield, MO (US); **Jack Q. Wilkinson**, Redwood City, CA (US)

Correspondence Address:
HOWREY SIMON ARNOLD & WHITE LLP
750 BERING DRIVE
HOUSTON, TX 77057 (US)

(73) Assignee: **Monsanto Technology LLC**

(21) Appl. No.: **10/427,169**

(22) Filed: **May 1, 2003**

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(60) Provisional application No. 60/171,173, filed on Dec. 16, 1999.

Publication Classification

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(52) **U.S. Cl.** **536/23.2**; 800/278

(57) **ABSTRACT**

The present invention relates to novel plant expression constructs. More specifically the present invention provides DNA constructs comprising 5' regulatory sequences for modulating the expression of operably linked genes in plants.

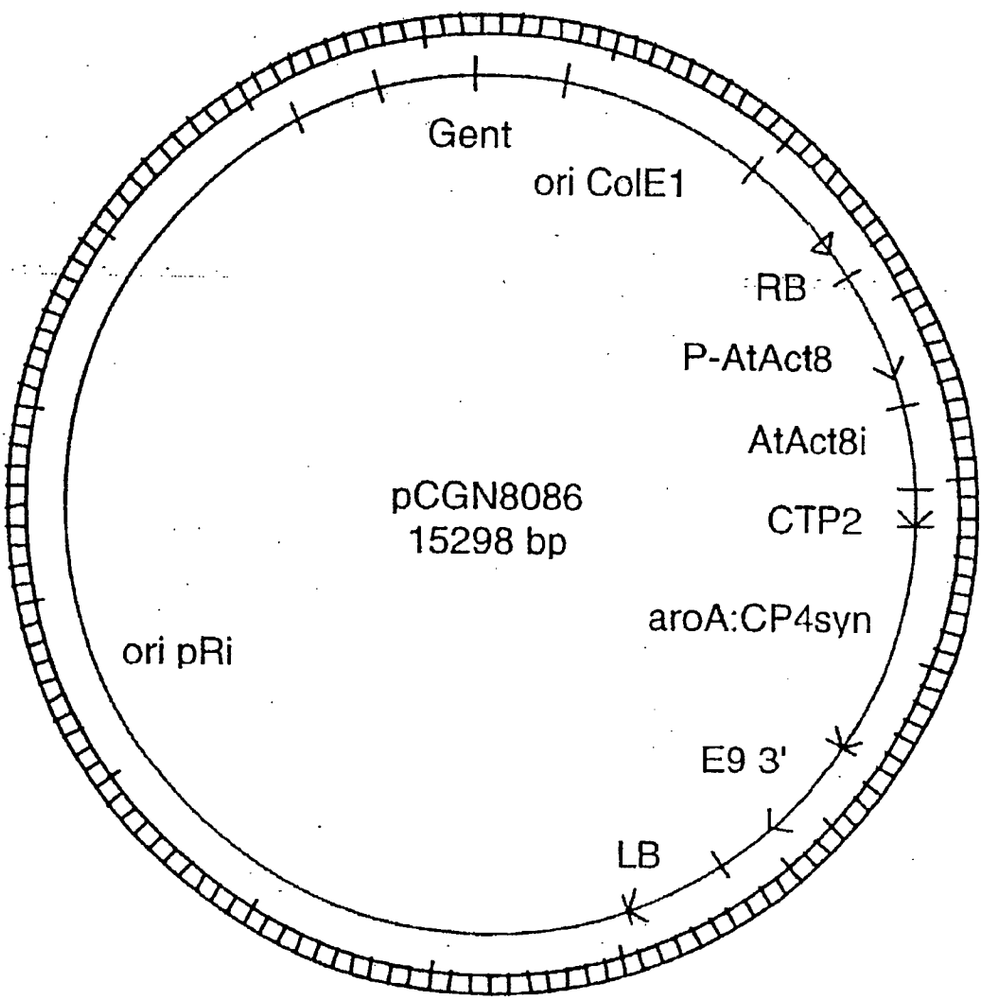

Gent
ori ColE1
RB
P-AtAct8
AtAct8i
pCGN8086
15298 bp
CTP2
aroA:CP4syn
ori pRi
E9 3'
LB

Figure 1

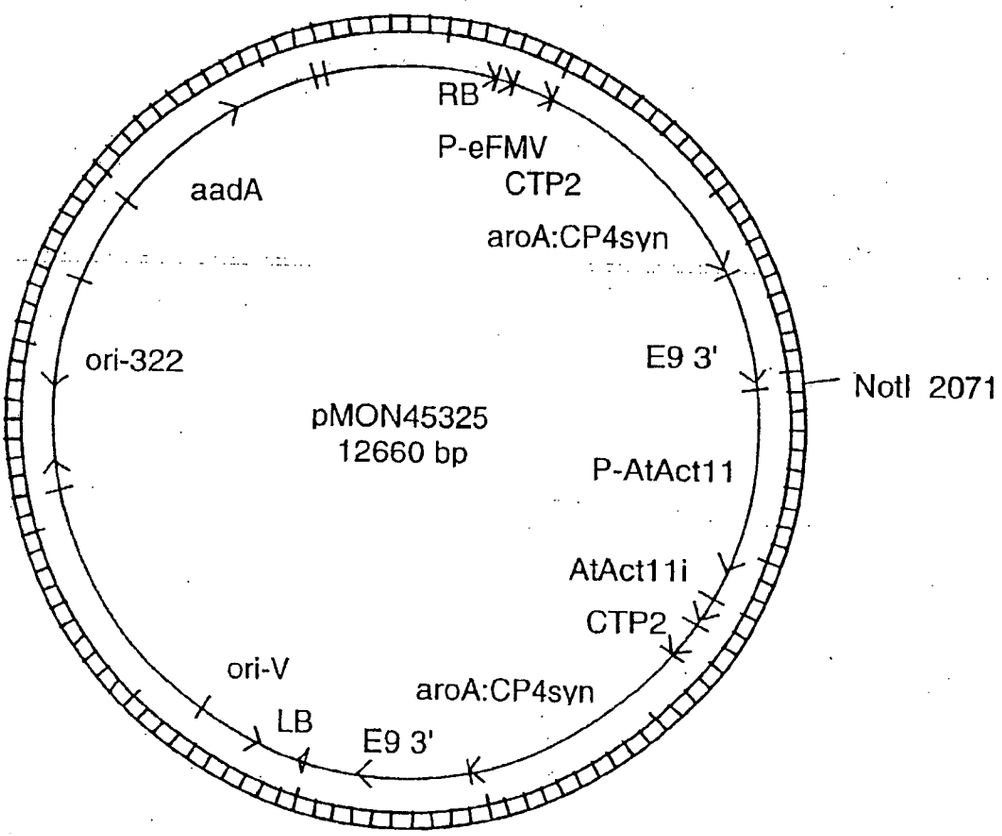


Figure 2

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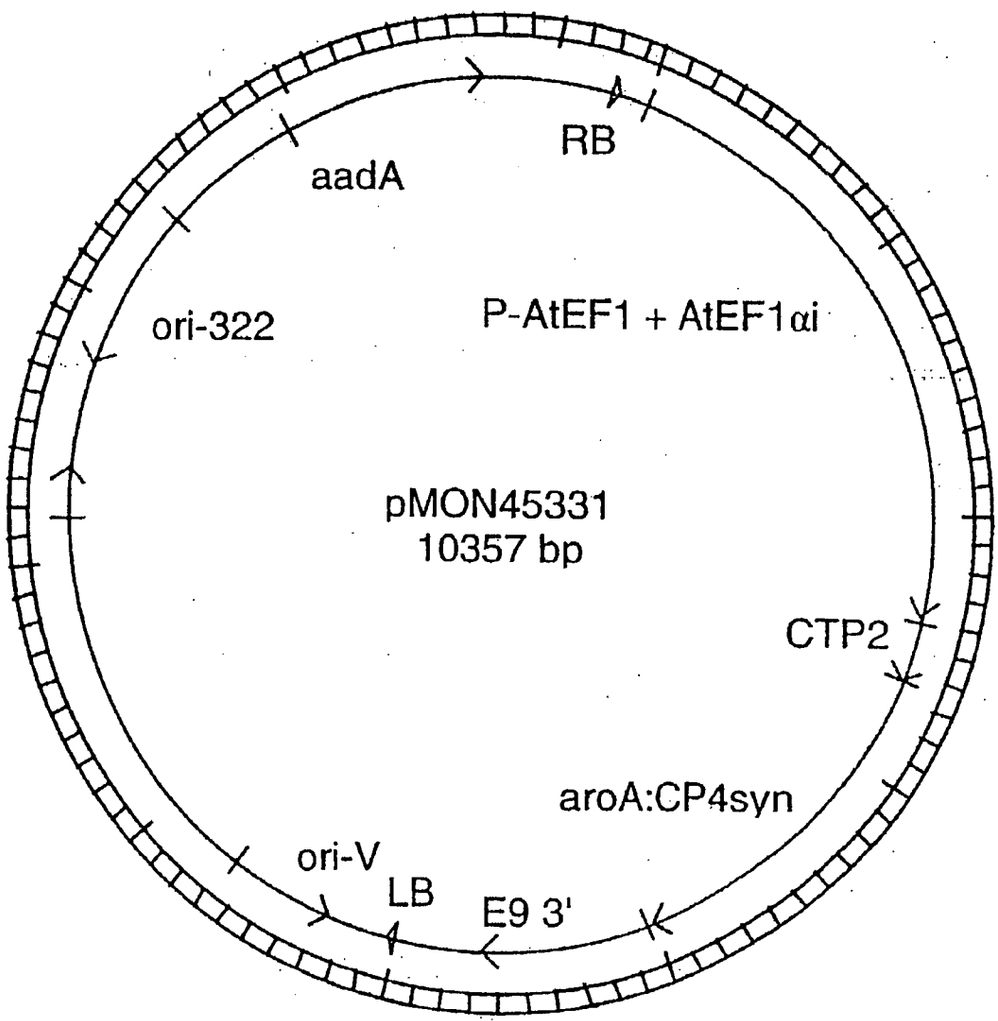

RB
aadA
ori-322
P-AtEF1 + AtEF1αi
pMON45331
10357 bp
CTP2
aroA:CP4syn
ori-V
LB
E9 3'

Figure 3

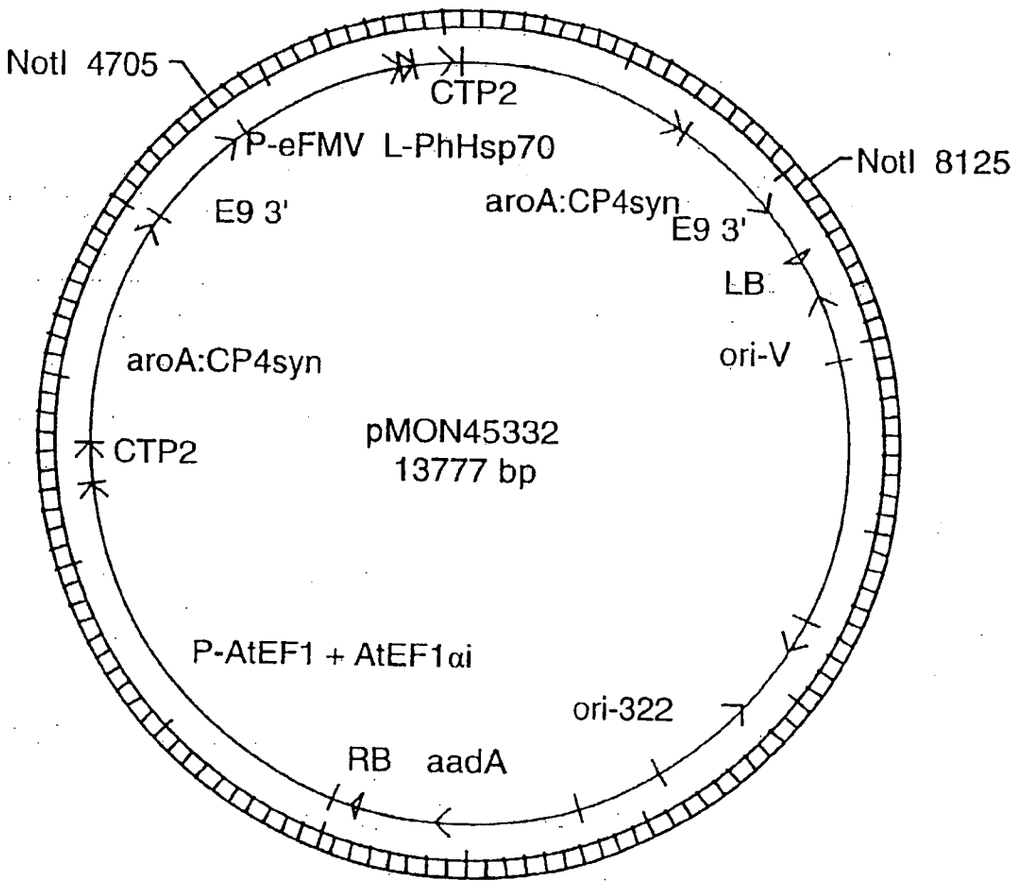


Figure 4

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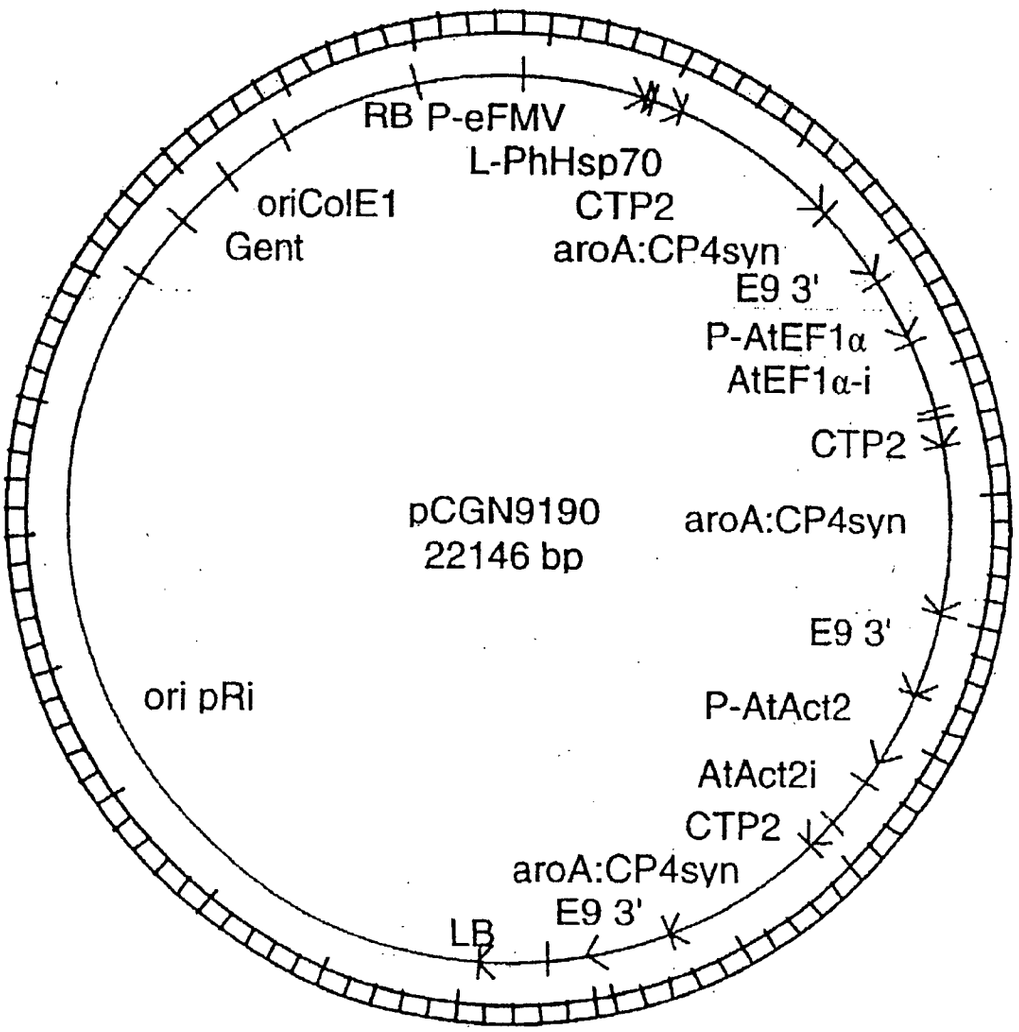

RB
P-eFMV
L-PhHsp70
CTP2
aroA:CP4syn
E9 3'
P-AtEF1α
AtEF1α-i
CTP2
aroA:CP4syn
E9 3'
P-AtAct2
AtAct2i
CTP2
aroA:CP4syn
E9 3'
LB
ori pRi
oriColE1
Gent
pCGN9190
22146 bp

Figure 5

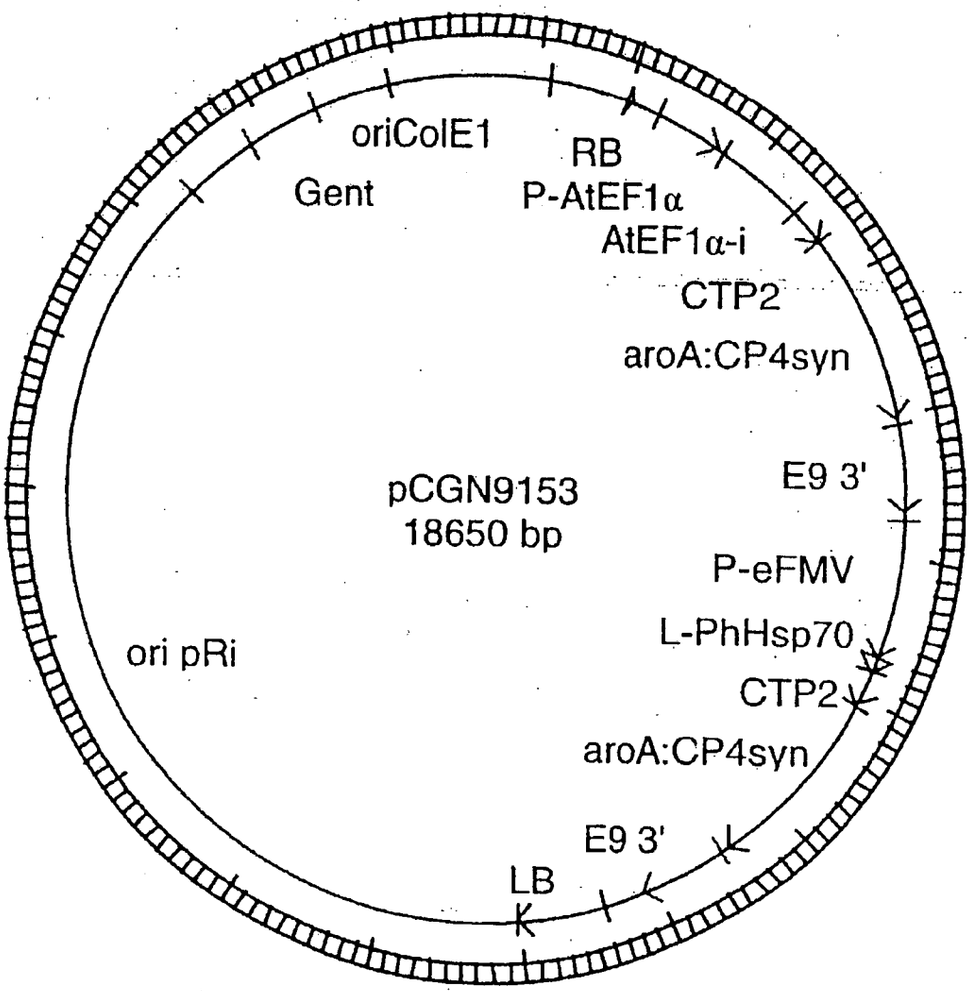


Figure 6

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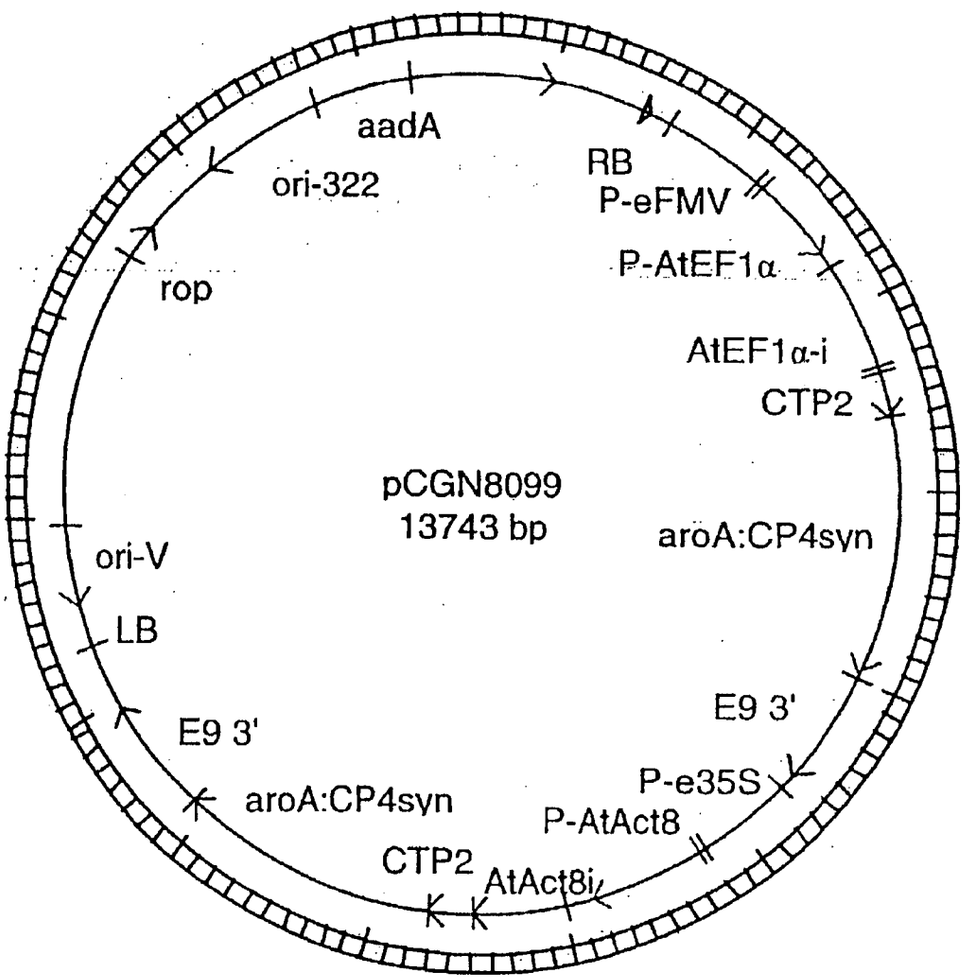

aadA
RB
ori-322
P-eFMV
P-AtEF1α
rop
AtEF1α-i
CTP2
pCGN8099
13743 bp
aroA:CP4syn
ori-V
LB
E9 3'
E9 3'
P-e35S
aroA:CP4syn
P-AtAct8
CTP2
AtAct8i

Figure 7

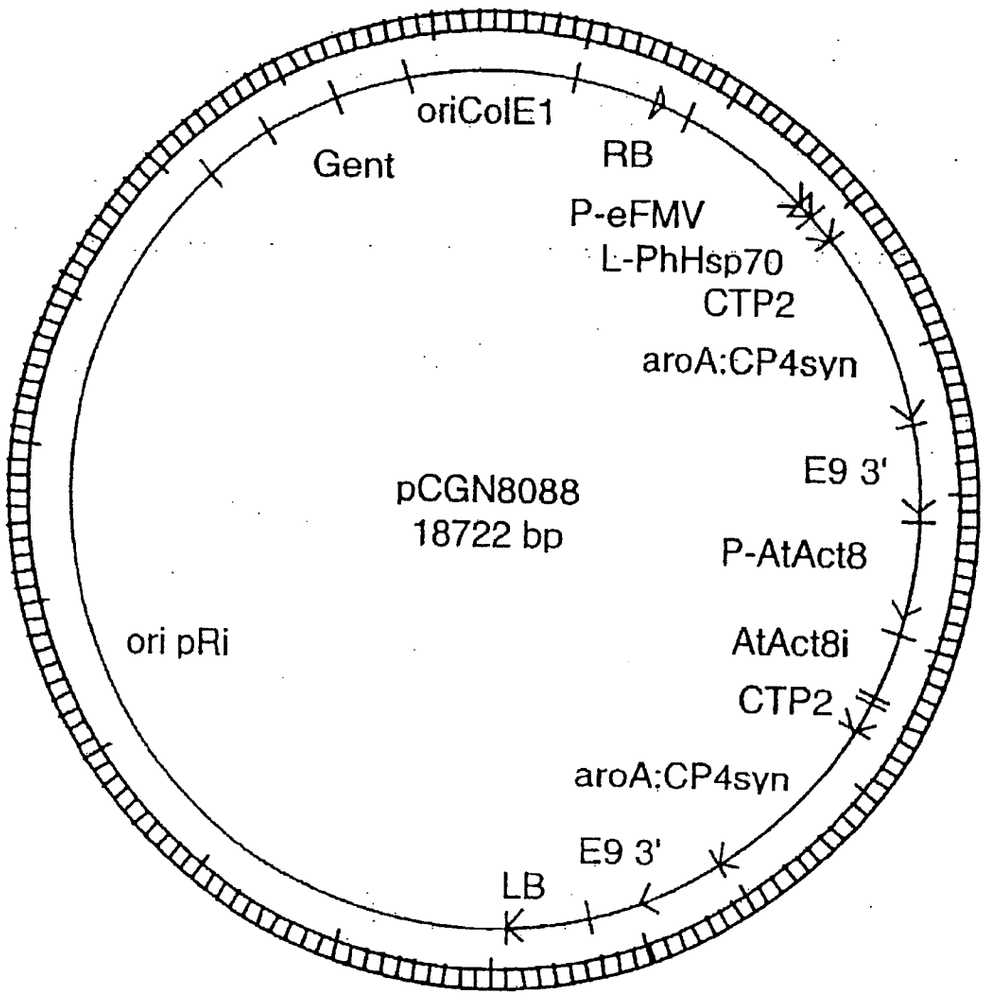


Figure 8

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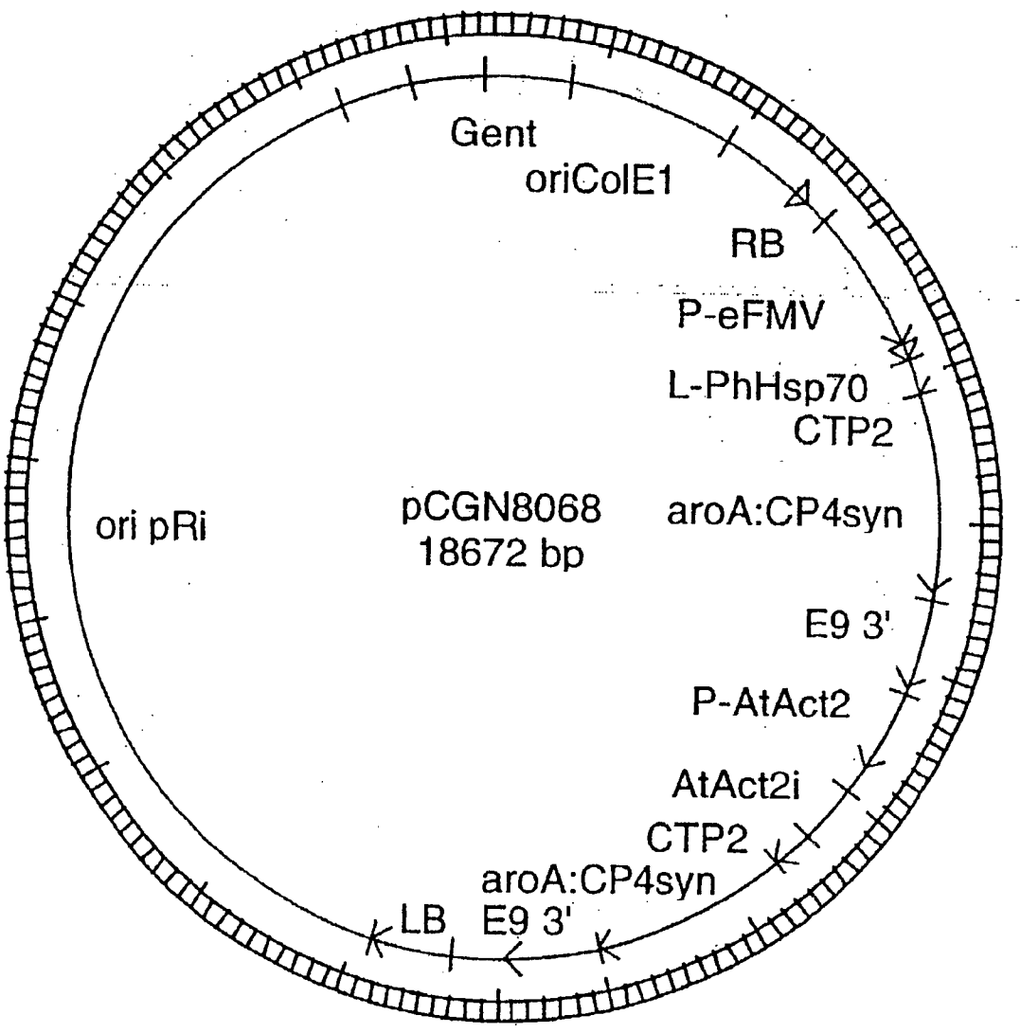

Gent
oriColE1
RB
P-eFMV
L-PhHsp70
CTP2
aroA:CP4syn
E9 3'
P-AtAct2
AtAct2i
CTP2
aroA:CP4syn
E9 3'
LB
ori pRi
pCGN8068
18672 bp

Figure 9.

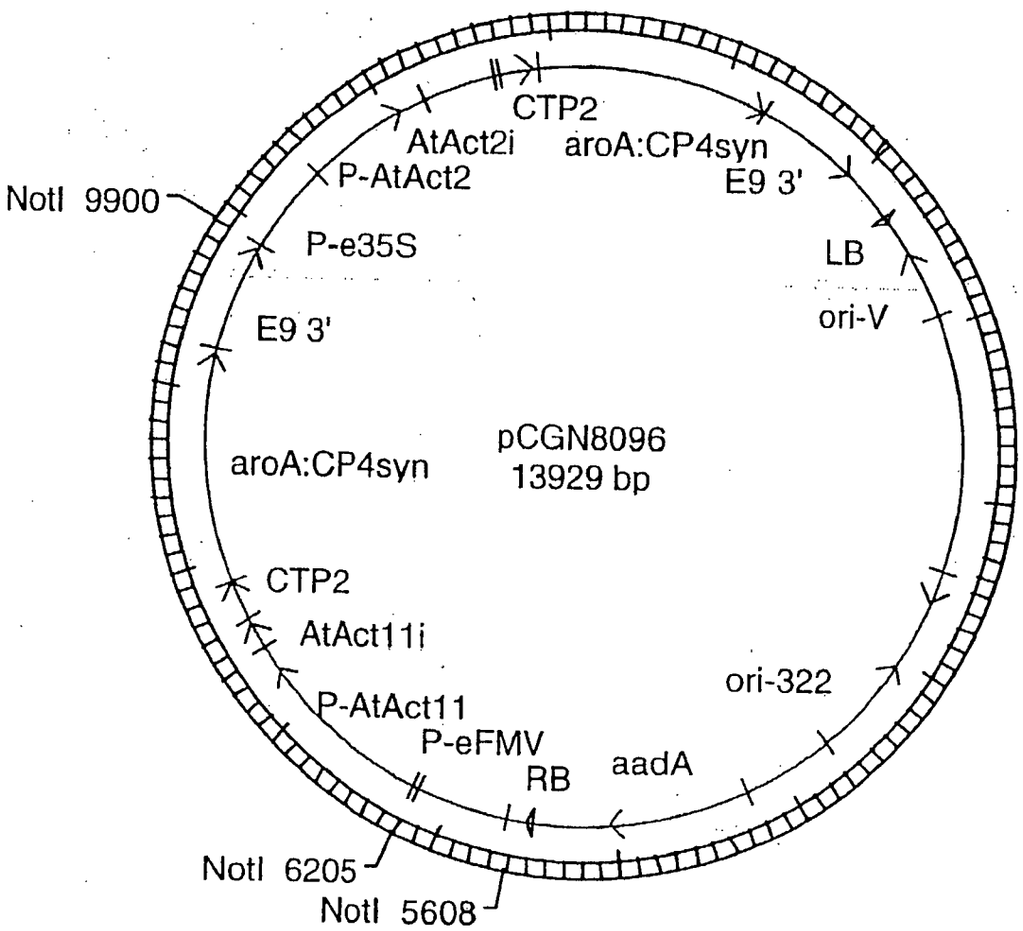


Figure 10

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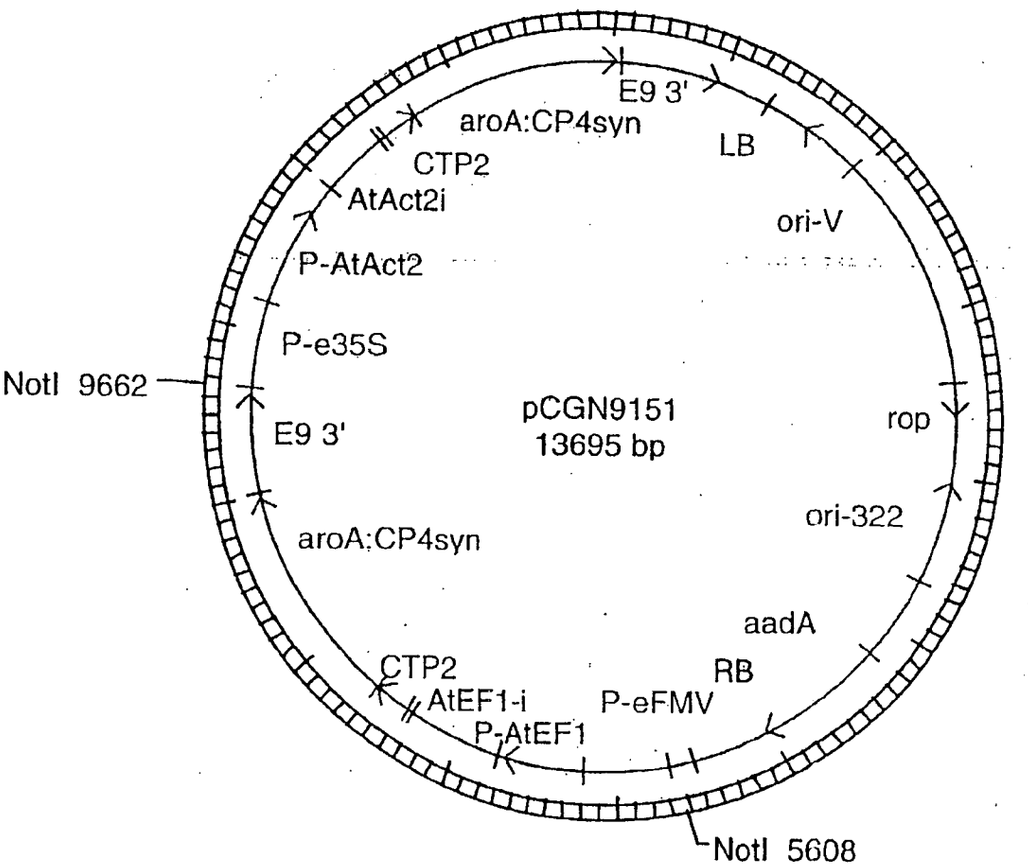

E9 3'
aroA:CP4syn
CTP2
AtAct2i
P-AtAct2
P-e35S
NotI 9662
E9 3'
aroA:CP4syn
CTP2
AtEF1-i
P-AtEF1
P-eFMV
RB
aadA
ori-322
rop
ori-V
LB
pCGN9151
13695 bp
NotI 5608

Figure 11

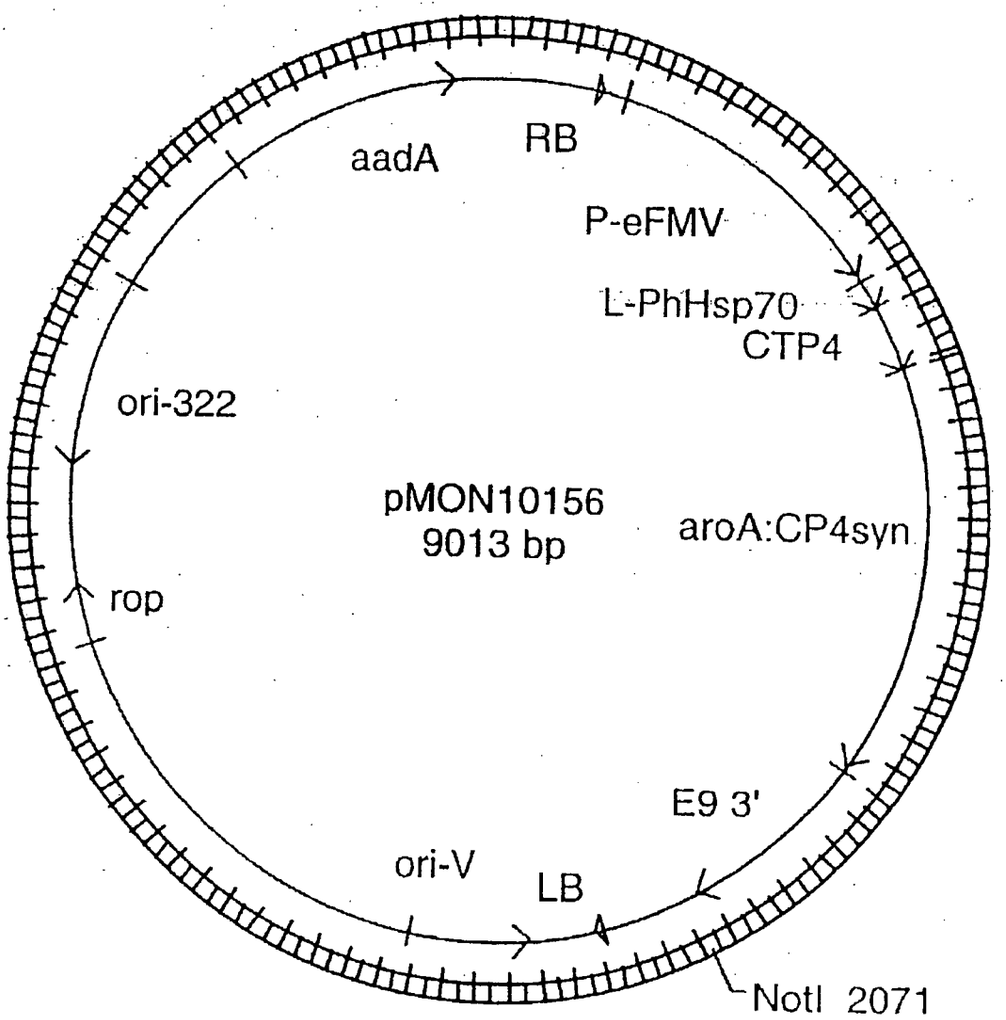


Figure 12

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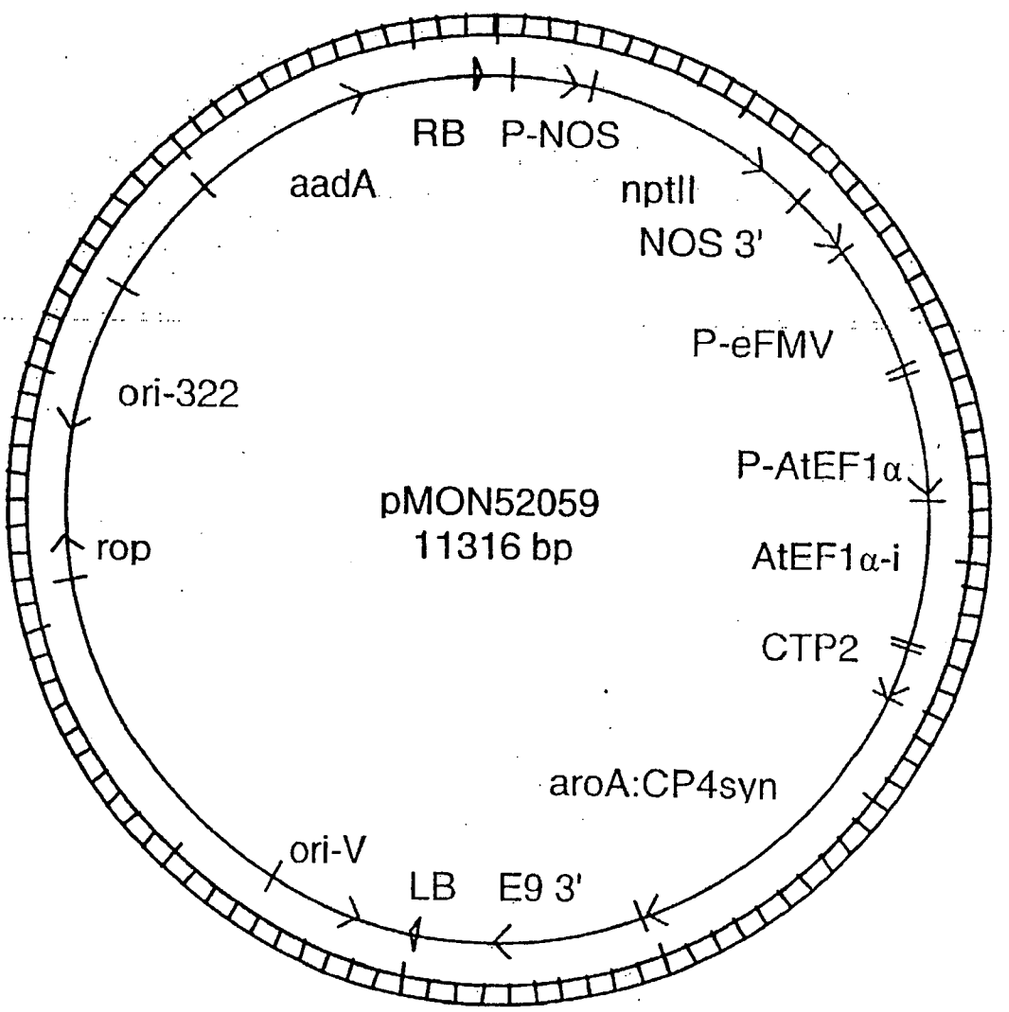

RB
P-NOS
aadA
nptII
NOS 3'
P-eFMV
ori-322
P-AtEF1α
pMON52059
11316 bp
rop
AtEF1α-i
CTP2
aroA:CP4syn
ori-V
LB
E9 3'

Figure 13

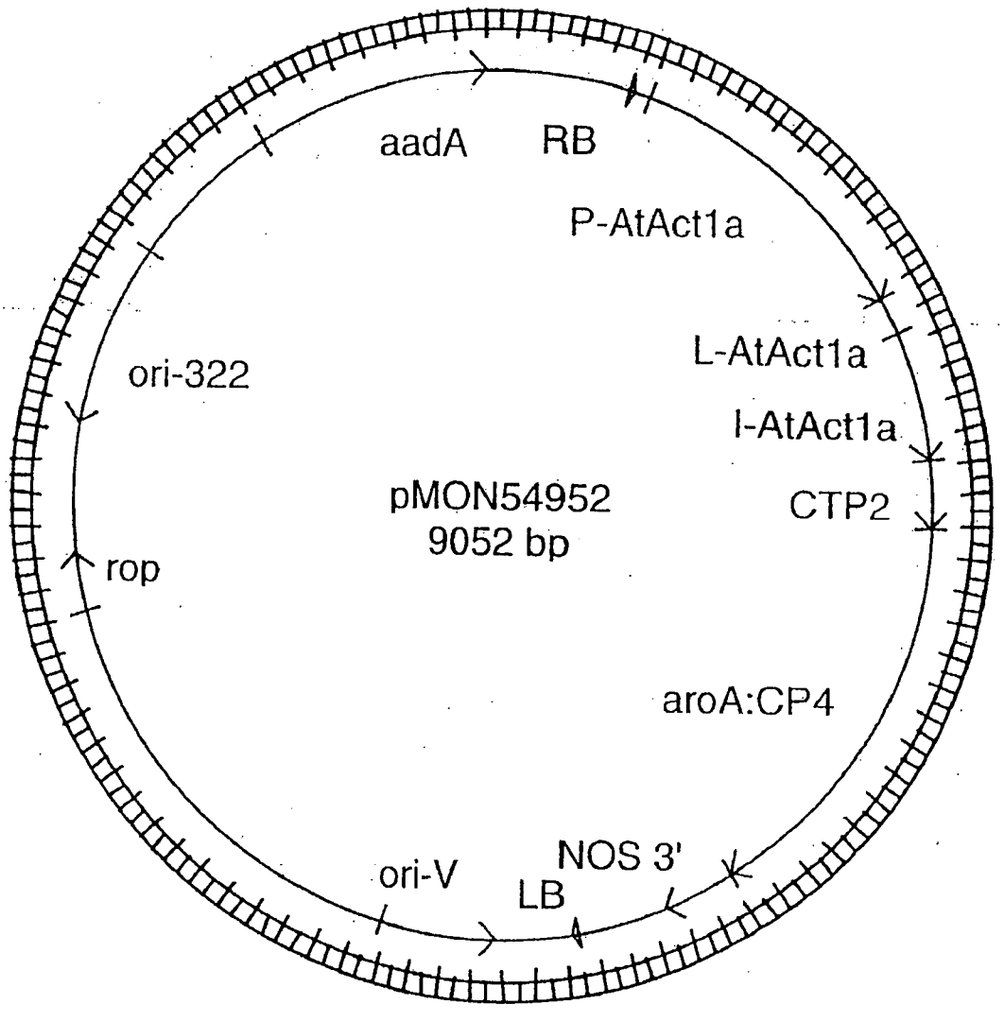


Figure 14

BNSDOCID: <US____20030199681A1_I_>

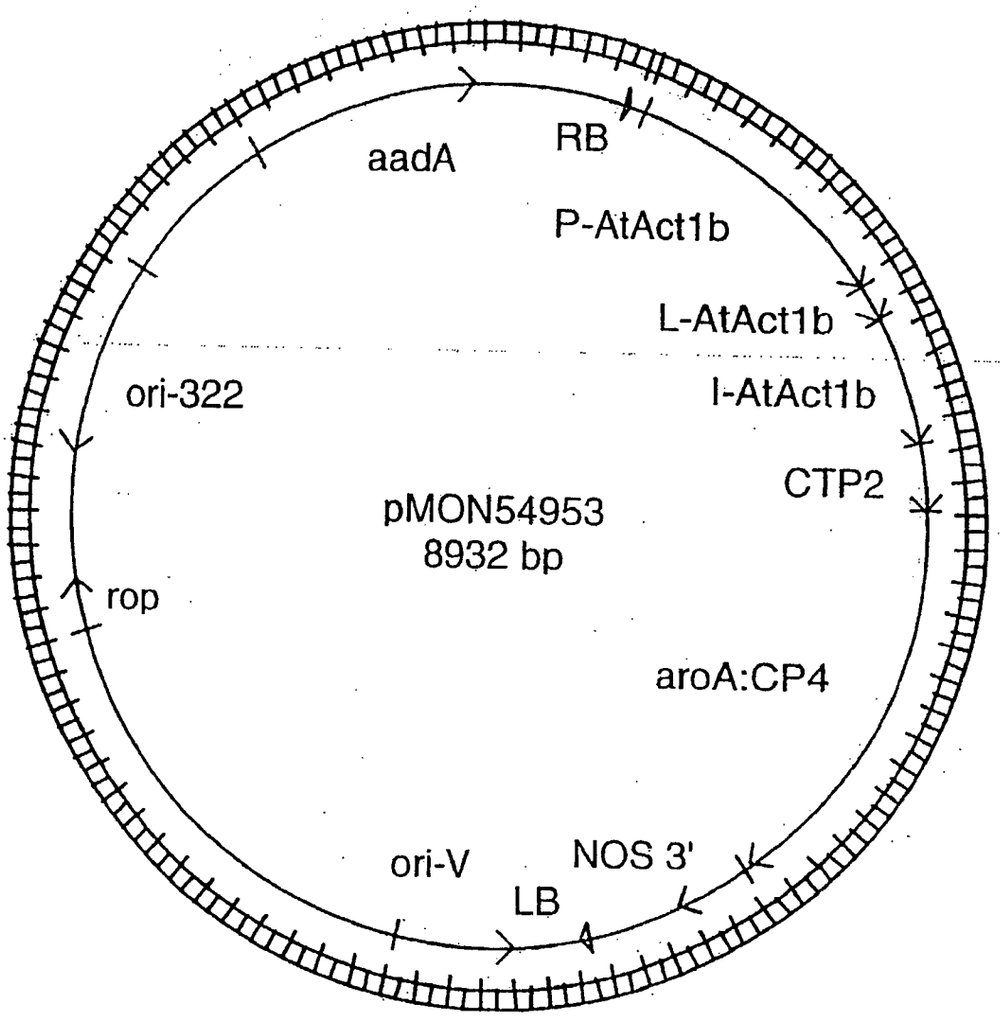

aadA
RB
P-AtAct1b
L-AtAct1b
I-AtAct1b
CTP2
aroA:CP4
NOS 3'
LB
ori-V
rop
ori-322
pMON54953
8932 bp

Figure 15

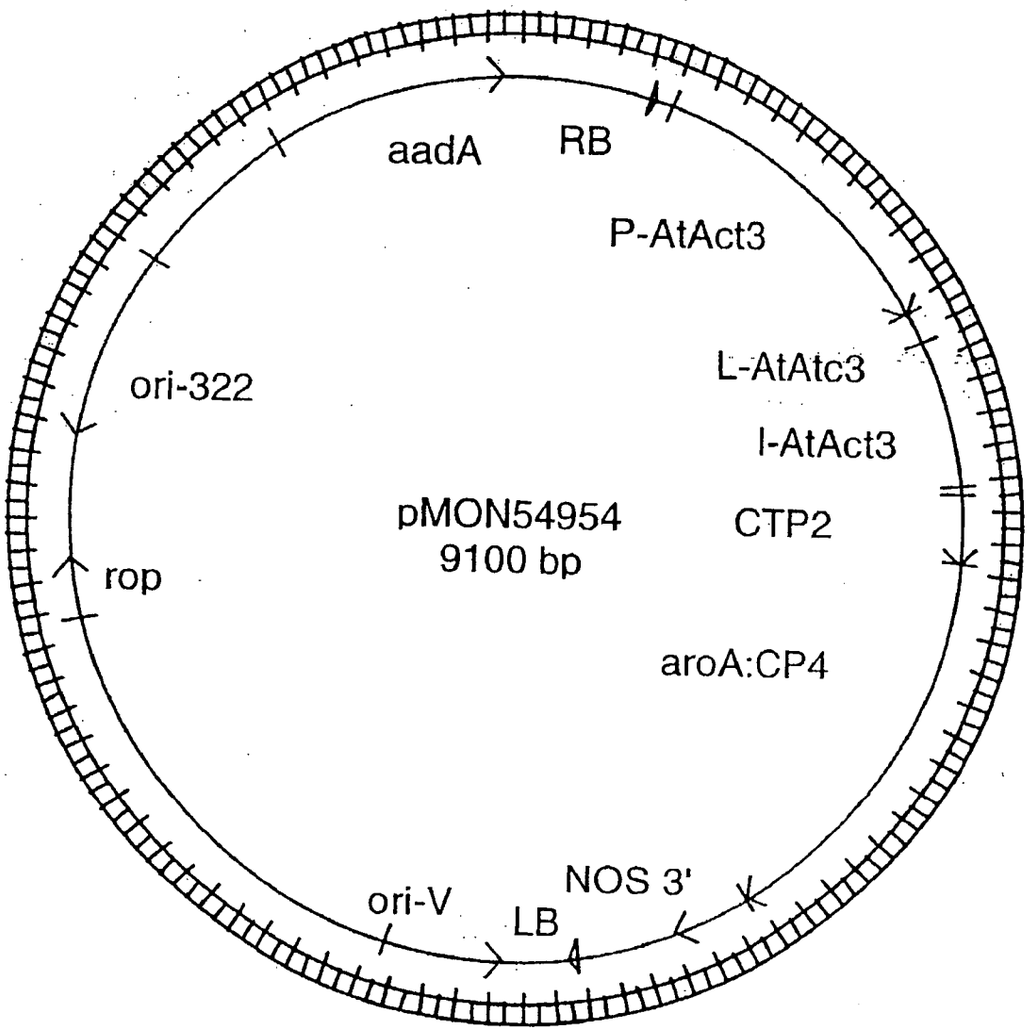


Figure 16

BNSDOCID: <US____20030199681A1_I_>

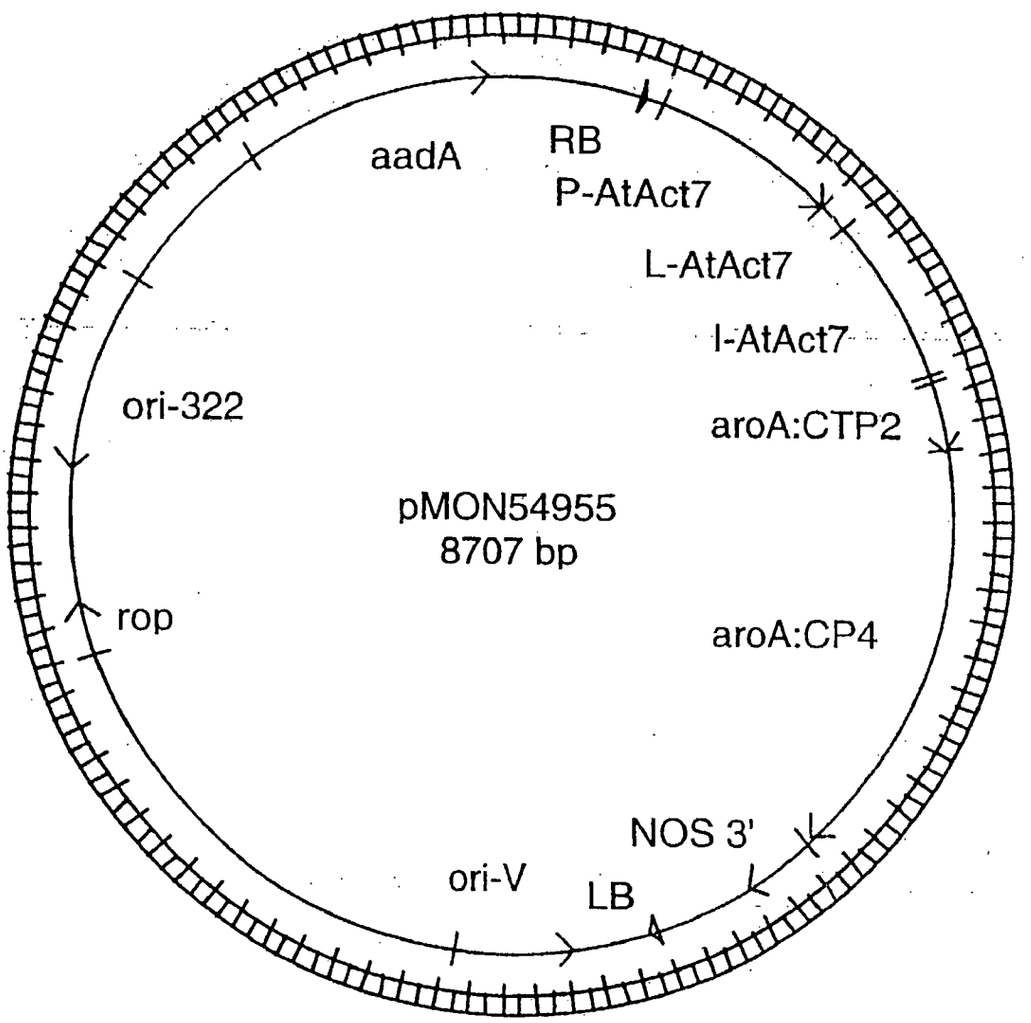

aadA
RB
P-AtAct7
L-AtAct7
I-AtAct7
aroA:CTP2
aroA:CP4
NOS 3'
LB
ori-V
rop
ori-322
pMON54955
8707 bp

Figure 17

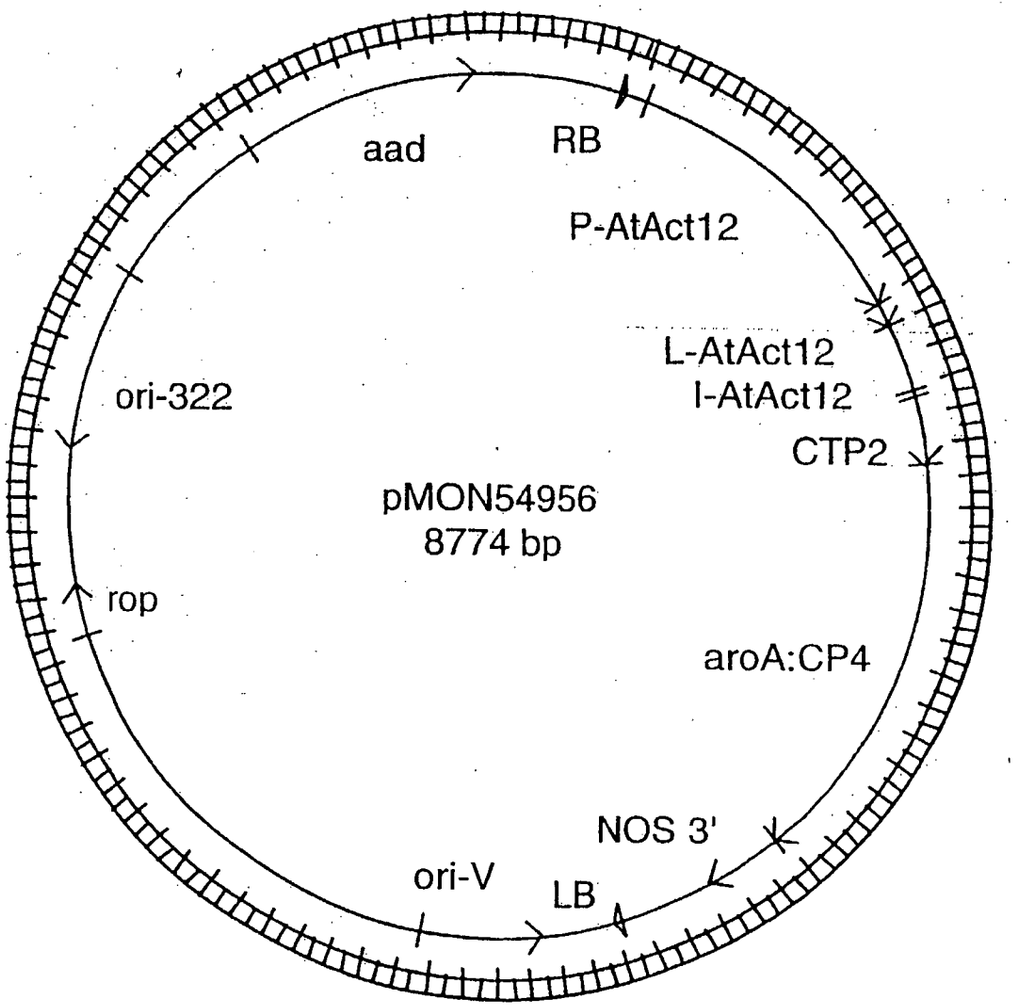


Figure 18

BNSDOCID: <US____20030199681A1_I_>

NOVEL PLANT EXPRESSION CONSTRUCTS

FIELD OF THE INVENTION

[0001] The present invention relates to the isolation and use of nucleic acid molecules for control of gene expression in plants, specifically novel plant promoters.

BACKGROUND OF THE INVENTION

[0002] One of the goals of plant genetic engineering is to produce plants with agronomically important characteristics or traits. Recent advances in genetic engineering have provided the requisite tools to produce transgenic plants that contain and express foreign genes (Kahl et al., World J. of Microbiol. Biotech. 11:449-460, 1995). Particularly desirable traits or qualities of interest for plant genetic engineering would include but are not limited to resistance to insects, fungal diseases, and other pests and disease-causing agents, tolerances to herbicides, enhanced stability or shelf-life, yield, environmental tolerances, and nutritional enhancements. The technological advances in plant transformation and regeneration have enabled researchers to take exogenous DNA, such as a gene or genes from a heterologous or a native source, and incorporate the exogenous DNA into the plant's genome. In one approach, expression of a novel gene that is not normally expressed in a particular plant or plant tissue may confer a desired phenotypic effect. In another approach, transcription of a gene or part of a gene in an antisense orientation may produce a desirable effect by preventing or inhibiting expression of an endogenous gene.

[0003] In order to produce a transgenic plant, a construct that includes a heterologous gene sequence that confers a desired phenotype when expressed in the plant is introduced into a plant cell. The construct also includes a plant promoter that is operably linked to the heterologous gene sequence, often a promoter not normally associated with the heterologous gene. The construct is then introduced into a plant cell to produce a transformed plant cell, and the transformed plant cell is regenerated into a transgenic plant. The promoter controls expression of the introduced DNA sequence to which the promoter is operably linked and thus affects the desired characteristic conferred by the DNA sequence.

[0004] It would be advantageous to have a variety of promoters to tailor gene expression such that a gene or gene(s) is transcribed efficiently at the right time during plant growth and development, in the optimal location in the plant, and in the amount necessary to produce the desired effect. For example, constitutive expression of a gene product may be beneficial in one location of the plant but less beneficial in another part of the plant. In other cases, it may be beneficial to have a gene product produced at a certain developmental stage of the plant or in response to certain environmental or chemical stimuli. The commercial development of genetically improved germplasm has also advanced to the stage of introducing multiple traits into crop plants, often referred to as a gene stacking approach. In this approach, multiple genes conferring different characteristics of interest can be introduced into a plant. It is important when introducing multiple genes into a plant that each gene is modulated or controlled for optimal expression and that the regulatory elements are diverse in order to reduce the potential of gene silencing. In light of these and other considerations, it is apparent that optimal control of gene expression and regulatory element diversity are important in plant biotechnology.

SUMMARY OF THE INVENTION

[0005] The present invention relates to DNA plant expression constructs that comprise Arabidopsis actin (Act) promoter sequences Act1a, Act1b, Act 2, Act3, Act7, Act8, Act11, Act12 and the elongation factor 1α (EF1α) promoter sequence, and fragments and cis elements derived from these promoters operably linked to heterologous structural gene sequences that function in crop plant cells.

[0006] Thus, according to one embodiment of the invention, a recombinant DNA construct is provided that comprises, in operable linkage, a promoter that is functional in a cell of a crop plant, the promoter comprising: at least one cis element derived from SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26; a structural DNA sequence heterologous to the promoter; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. For example, the promoter may consist essentially of a 5' regulatory region derived from any of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 (including or excluding any intron sequences located therein). The structural gene may comprise any heterologous nucleotide sequence wherein expression of the sequence results in an agronomically useful trait or product in a transgenic crop plant.

[0007] According to another aspect of the invention is a DNA construct comprising a structural DNA sequence operably linked to the promoter sequences of the present invention that encode a protein employed to confer herbicide tolerance to a crop plant. This herbicide tolerance protein includes, but is not limited to glyphosate tolerance protein genes such as a glyphosate resistant EPSP synthase gene alone, or in combination with one or more glyphosate degrading protein genes.

[0008] According to another embodiment of the invention, DNA constructs such as those described above are provided wherein the promoter is a hybrid or chimeric promoter comprising at least one cis element derived from one or more of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 operably linked to a heterologous promoter sequence such as a caulimovirus promoter, for example the Cauliflower mosaic virus 35S promoter or the Figwort mosaic virus promoter.

[0009] According to another embodiment of the invention, DNA constructs, such as those described above, are provided in tandem, wherein the promoter is a hybrid or chimeric promoter comprising at least one cis element derived from one or more of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 operably linked to a heterologous gene sequence that expresses in transgenic crop plant cells. The chimeric promoter sequences more specifically comprising the sequences identified in SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30.

[0010] According to another embodiment of the invention, a DNA construct such as that described above is provided

wherein the structural DNA sequence is a glyphosate tolerance gene, such that when the DNA construct is introduced into a plant cell, it confers to the plant cell tolerance to an aqueous glyphosate formulation that includes at least 50 grams acid equivalent per liter (g a.e./l) of glyphosate. In other related embodiments, the DNA construct confers to the plant cell tolerance to glyphosate formulations having higher glyphosate concentrations (for example, at least 300 grams acid equivalent per liter of glyphosate. According to one embodiment, the DNA construct confers to the plant cell tolerance to at least one application of Roundup Ultra® at a rate of 16 ounces (oz) per acre, for example, and in other embodiments, glyphosate tolerance extends to one to two or more applications of 16 oz per acre, 32 oz per acre, or 64 oz per acre, for example.

[0011] According to another embodiment of the invention, transgenic crop plants are provided that are transformed with a DNA construct as described above, including monocot species and dicot species. We have discovered that the Arabidopsis actin and Arabidopsis EF1α promoters are sufficiently active in other crop plant species such as cotton, tomato, and sunflower, for example, that when used to control expression of a glyphosate tolerance gene, such as aroA:CP4, the plants tolerate commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

[0012] According to another embodiment of the invention, transgenic crop plants are provided that are transformed with multiple DNA constructs comprising the Arabidopsis actin and Arabidopsis EF1α promoters are sufficiently active in other plant species such as cotton, tomato, sunflower, for example, that when used to control expression of a glyphosate tolerance gene such as aroA:CP4, the plants tolerated commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

[0013] According to another embodiment of the invention, transgenic crop plants are provided that are transformed with DNA constructs comprising the Arabidopsis actin and Arabidopsis EF1α promoters as chimeric DNA molecules in fusion with caulimovirus DNA molecules having promoter activity in plants sufficiently active in other plant species such as cotton, tomato, canola, soybean, and sunflower, for example, that when used to control expression of a glyphosate tolerance gene such as aroA:CP4, the plants tolerate commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

[0014] According to another embodiment of the invention methods are provided for expressing a structural DNA sequence in a plant. Such methods comprise, providing a DNA construct as described above, introducing the DNA construct into a plant cell, and regenerating the plant cell to produce a plant such that the structural DNA is expressible in the plant. According to a related embodiment, a method of controlling weeds is provided in which the DNA construct comprises a glyphosate tolerance gene and one applies to a crop plant transformed with the DNA construct an amount of glyphosate that is sufficient to control weeds without significantly damaging the crop plant.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **FIG. 1** is a plasmid map of pCGN8086

[0016] **FIG. 2** is a plasmid map of pMON45325

[0017] **FIG. 3** is a plasmid map of pMON45331

[0018] **FIG. 4** is a plasmid map of pMON45332

[0019] **FIG. 5** is a plasmid map of pCGN9190

[0020] **FIG. 6** is a plasmid map of pCGN9153

[0021] **FIG. 7** is a plasmid map of pCGN8099

[0022] **FIG. 8** is a plasmid map of pCGN8088

[0023] **FIG. 9** is a plasmid map of pCGN8068

[0024] **FIG. 10** is a plasmid map of pCGN8096

[0025] **FIG. 11** is a plasmid map of pCGN9151

[0026] **FIG. 12** is a plasmid map of pMON10156

[0027] **FIG. 13** is a plasmid map of pMON52059

[0028] **FIG. 14** is a plasmid map of pMON54952

[0029] **FIG. 15** is a plasmid map of pMON54953

[0030] **FIG. 16** is a plasmid map of pMON54954

[0031] **FIG. 17** is a plasmid map of pMON54955

[0032] **FIG. 18** is a plasmid map of pMON54956

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

[0033] SEQ ID NO:1 is the forward PCR primer used for the isolation of the Act2 promoter

[0034] SEQ ID NO:2 is the reverse PCR primer used for the isolation of the Act2 promoter

[0035] SEQ ID NO:3 is the forward PCR primer used for the isolation of the Act8 promoter

[0036] SEQ ID NO:4 is the reverse PCR primer used for the isolation of the Act8 promoter

[0037] SEQ ID NO:5 is the forward PCR primer used for the isolation of the Act11 promoter

[0038] SEQ ID NO:6 is the reverse PCR primer used for the isolation of the Act11 promoter

[0039] SEQ ID NO:7 is the forward PCR primer used for the isolation of the EF1 promoter

[0040] SEQ ID NO:8 is the reverse PCR primer used for the isolation of the EF1 promoter

[0041] SEQ ID NO:9 is the sequence of the Act2 promoter including the intron sequence of the Act2 gene. Base positions 1-764 represent the promoter sequence; base positions 765-1215 represent the intron followed by 5 bases of 5' untranslated region (5' UTR) prior to the ATG; the transcription start site is located at base position 597.

[0042] SEQ ID NO:10 is the sequence of the Act8 promoter including the first intron of the Act8 gene. Base positions 1-797 represent the promoter sequence; base positions 798-1259 represent the intron followed by 10 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 646.

[0043] SEQ ID NO:11 is the sequence of the Act11 promoter including the first intron of the Act11 gene. Base positions 1-1218 represent the promoter sequence; base positions 1219-1381 represent the intron followed by 10 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 1062.

[0044] SEQ ID NO:12 is the sequence of the EF1 promoter including the first intron of the EF1 gene. Base positions 1-536 represent the promoter sequence; base positions 537-1137 represent the intron followed by 22 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 481.

[0045] SEQ ID NO:13 is the forward PCR primer used for the isolation of the Act1a promoter

[0046] SEQ ID NO:14 is the forward PCR primer used for the isolation of the Act1b promoter

[0047] SEQ ID NO:15 is the reverse PCR primer used for the isolation of the Act1a and Act1b promoter

[0048] SEQ ID NO:16 is the forward PCR primer used for the isolation of the Act3 promoter

[0049] SEQ ID NO:17 is the reverse PCR primer used for the isolation of the Act3 promoter

[0050] SEQ ID NO:18 is the forward PCR primer used for the isolation of the Act7 promoter

[0051] SEQ ID NO:19 is the reverse PCR primer used for the isolation of the Act7 promoter

[0052] SEQ ID NO:20 is the forward PCR primer used for the isolation of the Act12 promoter

[0053] SEQ ID NO:21 is the reverse PCR primer used for the isolation of the Act12 promoter

[0054] SEQ ID NO:22 is the sequence of the Act1a promoter including the first intron of the Act1a gene. Base positions 1-1033 represent the promoter sequence; base positions 1034-1578 represent the intron and 5' UTR.

[0055] SEQ ID NO:23 is the sequence of the Act1b promoter including the first intron of the Act1b gene. Base positions 1-914 represent the promoter sequence; base positions 915-1468 represent the intron and 5' UTR sequence.

[0056] SEQ ID NO:24 is the sequence of the Act3 promoter including the first intron of the Act3 gene. Base positions 1-1023 represent the promoter sequence; base positions 1024-1642 represent the intron and 5' UTR sequence.

[0057] SEQ ID NO:25 is the sequence of the Act7 promoter including the first intron of the Act7 gene. Base positions 1-600 represent the promoter sequence; base positions 601-1241 represent the intron and 5' UTR sequence.

[0058] SEQ ID NO:26 is the sequence of the Act 12 promoter including the first intron of the Act12 gene. Base positions 1-1017 represent the promoter sequence; base positions 1018-1313 represent the intron and 5' UTR sequence.

[0059] SEQ ID NO:27 is the sequence of the chimeric FMV-Act11 promoter including the first intron of the Act11 gene. Base positions 1-536 represent the duplicated FMV promoter sequence; base positions 553-1946 represent the Arabidopsis Actin 11 promoter, intron and 5' UTR sequence.

[0060] SEQ ID NO:28 is the sequence of the chimeric FMV-EF1α promoter including the first intron of the EF1α gene. Base positions 1-536 present the FMV promoter sequence; base positions 553-1695 represent the EF1α promoter, intron and 5' UTR sequence.

[0061] SEQ ID NO:29 is the sequence of the CaMV-Act8 promoter including the first intron of the Act8 gene. Base positions 1-523 present the CaMV promoter sequence; base positions 534-1800 represent the Act8 promoter, intron and 5' UTR sequence.

[0062] SEQ ID NO:30 is the sequence of the CaMV-Act2 promoter including the first intron of the Act2 gene. Base positions 1-523 represent the CaMV promoter sequence; base positions 534-1742 represent the Act2 promoter, intron and 5' UTR sequence.

DETAILED DESCRIPTION OF THE INVENTION

[0063] This application claims the benefit of U.S. Provisional Application No. 60/171,173, filed Dec. 16, 1999. The following definitions and methods are provided to better define, and to guide those of ordinary skill in the art in the practice of, the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used. The standard one- and three-letter nomenclature for amino acid residues is used. "Nucleic acid (sequence)" or "polynucleotide (sequence)" refers to single- or double-stranded DNA or RNA of genomic or synthetic origin, i.e., a polymer of deoxyribonucleotide or ribonucleotide bases, respectively, read from the 5' (upstream) end to the 3' (downstream) end. The nucleic acid can represent the sense or complementary (antisense) strand.

[0064] "Native" refers to a naturally occurring ("wild-type") nucleic acid sequence.

[0065] "Heterologous" sequence refers to a sequence which originates from a foreign source or species or, if from the same source, is modified from its original form.

[0066] An "isolated" nucleic acid sequence is substantially separated or purified away from other nucleic acid sequences with which the nucleic acid is normally associ-

ated in the cell of the organism in which the nucleic acid naturally occurs, i.e., other chromosomal or extrachromosomal DNA. The term embraces nucleic acids that are biochemically purified so as to substantially remove contaminating nucleic acids and other cellular components. The term also embraces recombinant nucleic acids and chemically synthesized nucleic acids. The term "substantially purified", as used herein, refers to a molecule separated from other molecules normally associated with it in its native state. More preferably, a substantially purified molecule is the predominant species present in a preparation. A substantially purified molecule may be greater than 60% free, preferably 75% free, more preferably 90% free from the other molecules (exclusive of solvent) present in the natural mixture. The term "substantially purified" is not intended to encompass molecules present in their native state.

[0067] A first nucleic acid sequence displays "substantially identity" to a reference nucleic acid sequence if, when optimally aligned (with appropriate nucleotide insertions or deletions totaling less than 20 percent of the reference sequence over the window of comparison) with the other nucleic acid (or its complementary strand), there is at least about 75% nucleotide sequence identity, preferably at least about 80% identity, more preferably at least about 85% identity, and most preferably at least about 90% identity over a comparison window of at least 20 nucleotide positions, preferably at least 50 nucleotide positions, more preferably at least 100 nucleotide positions, and most preferably over the entire length of the first nucleic acid. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482, 1981; by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443, 1970; by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; preferably by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA) in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis. The reference nucleic acid may be a full-length molecule or a portion of a longer molecule. Alternatively, two nucleic acids are have substantial identity if one hybridizes to the other under stringent conditions, as defined below.

[0068] A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the sequences are so arranged that the first nucleic acid sequence affects the function of the second nucleic-acid sequence. Preferably, the two sequences are part of a single contiguous nucleic acid molecule and more preferably are adjacent. For example, a promoter is operably linked to a gene if the promoter regulates or mediates transcription of the gene in a cell.

[0069] A "recombinant" nucleic acid is made by an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. Techniques for nucleic-acid manipulation are well-known (see for example Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press, 1995; Birren et al., Genome Analysis: volume 1, Analyzing DNA, (1997), volume 2, Detecting Genes, (1998), volume 3, Cloning Systems, (1999) volume 4, Mapping Genomes, (1999), Cold Spring Harbor, N.Y.).

[0070] Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci et al., J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers.

[0071] A "synthetic nucleic acid sequence" can be designed and chemically synthesized for enhanced expression in particular host cells and for the purposes of cloning into appropriate constructs. Host cells often display a preferred pattern of codon usage (Murray et al., 1989). Synthetic DNAs designed to enhance expression in a particular host should therefore reflect the pattern of codon usage in the host cell. Computer programs are available for these purposes including but not limited to the "BestFit" or "Gap" programs of the Sequence Analysis Software Package, Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, Wis. 53711.

[0072] "Amplification" of nucleic acids or "nucleic acid reproduction" refers to the production of additional copies of a nucleic acid sequence and is carried out using polymerase chain reaction (PCR) technologies. A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in PCR Protocols: A Guide to Methods and Applications, ed. Innis et al., Academic Press, San Diego, 1990. In PCR, a primer refers to a short oligonucleotide of defined sequence which is annealed to a DNA template to initiate the polymerase chain reaction.

[0073] "Transformed", "transfected", or "transgenic" refers to a cell, tissue, organ, or organism into which has been introduced a foreign nucleic acid, such as a recombinant construct. Preferably, the introduced nucleic acid is integrated into the genomic DNA of the recipient cell, tissue, organ or organism such that the introduced nucleic acid is inherited by subsequent progeny. A "transgenic" or "transformed" cell or organism also includes progeny of the cell or organism and progeny produced from a breeding program employing such a "transgenic" plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a recombinant construct or construct.

[0074] The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression. Some genes can be transcribed into mRNA and translated into polypeptides (structural genes); other genes can be transcribed into RNA (e.g. rRNA, tRNA); and other types of gene function as regulators of expression (regulator genes).

[0075] "Expression" of a gene refers to the transcription of a gene to produce the corresponding MRNA and translation of this mRNA to produce the corresponding gene product, i.e., a peptide, polypeptide, or protein. Gene expression is controlled or modulated by regulatory elements including 5' regulatory elements such as promoters.

[0076] "Genetic component" refers to any nucleic acid sequence or genetic element which may also be a component

or part of an expression construct. Examples of genetic components include, but are not limited to promoter regions, 5' untranslated leaders, introns; genes, 3' untranslated regions, and other regulatory sequences or sequences which affect transcription or translation of one or more nucleic acid sequences.

[0077] The terms "recombinant DNA construct", "recombinant construct", "expression construct" or "expression cassette" refer to any agent such as a plasmid, cosmid, virus, BAC (bacterial artificial chromosome), autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a DNA molecule in which one or more DNA sequences have been linked in a functionally operative manner using well-known recombinant DNA techniques.

[0078] "Complementary" refers to the natural association of nucleic acid sequences by base-pairing (A-G-T pairs with the complementary sequence T-C-A). Complementarity between two single-stranded molecules may be partial, if only some of the nucleic acids pair are complementary; or complete, if all bases pair are complementary. The degree of complementarity affects the efficiency and strength of hybridization and amplification reactions.

[0079] "Homology" refers to the level of similarity between nucleic acid or amino acid sequences in terms of percent nucleotide or amino acid positional identity, respectively, i.e., sequence similarity or identity. Homology also refers to the concept of similar functional properties among different nucleic acids or proteins.

[0080] "Promoter" refers to a nucleic acid sequence located upstream or 5' to a translational start codon of an open reading frame (or protein-coding region) of a gene and that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) to initiate transcription. A "plant promoter" is a native or non-native promoter that is functional in plant cells. Constitutive promoters are functional in most or all tissues of a plant throughout plant development. Tissue-, organ- or cell-specific promoters are expressed only or predominantly in a particular tissue, organ, or cell type, respectively. Rather than being expressed "specifically" in a given tissue, organ, or cell type, a promoter may display "enhanced" expression, i.e., a higher level of expression, in one part (e.g., cell type, tissue, or organ) of the plant compared to other parts of the plant. Temporally regulated promoters are functional only or predominantly during certain periods of plant development or at certain times of day, as in the case of genes associated with circadian rhythm, for example. Inducible promoters selectively express an operably linked DNA sequence in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters regulated by light, heat, stress, flooding or drought, phytohormones, wounding, or chemicals such as ethanol, jasmonate, salicylic acid, or safeners.

[0081] Any plant promoter can be used as a 5' regulatory sequence for modulating expression of a particular gene or genes. One preferred promoter would be a plant RNA polymerase II promoter. Plant RNA polymerase II promoters, like those of other higher eukaryotes, have complex structures and are comprised of several distinct elements. One such element is the TATA box or Goldberg-Hogness box, which is required for correct expression of eukaryotic genes in vitro and accurate, efficient initiation of transcription in vivo. The TATA box is typically positioned at approximately −25 to −35, that is, at 25 to 35 basepairs (bp) upstream (5') of the transcription initiation site, or cap site, which is defined as position +1 (Breathnach and Chambon, Ann. Rev. Biochem. 50:349-383, 1981; Messing et al., In: Genetic Engineering of Plants, Kosuge et al., eds., pp. 211-227, 1983). Another common element, the CCAAT box, is located between −70 and −100 bp. In plants, the CCAAT box may have a different consensus sequence than the functionally analogous sequence of mammalian promoters (the plant analogue has been termed the "AGGA box" to differentiate it from its animal counterpart; Messing et al., In: Genetic Engineering of Plants, Kosuge et al., eds., pp. 211-227, 1983). In addition, virtually all promoters include additional upstream activating sequences or enhancers (Benoist and Chambon, Nature 290:304-310, 1981; Gruss et al., Proc. Nat. Acad. Sci. USA 78:943-947, 1981; and Khoury and Gruss, Cell 27:313-314, 1983) extending from around −100 bp to −1,000 bp or more upstream of the transcription initiation site.

[0082] When fused to heterologous DNA sequences, such promoters typically cause the fused sequence to be transcribed in a manner that is similar to that of the gene sequence with which the promoter is normally associated. Promoter fragments that include regulatory sequences can be added (for example, fused to the 5' end of, or inserted within, an active promoter having its own partial or complete regulatory sequences (Fluhr et al., Science 232:1106-1112, 1986; Ellis et al., EMBO J. 6:11-16, 1987; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986-8990, 1987; Poulsen and Chua, Mol. Gen. Genet. 214:16-23, 1988; Comai et al., Plant Mol. Biol. 15:373-381, 1991). Alternatively, heterologous regulatory sequences can be added to the 5' upstream region of an inactive, truncated promoter, e.g., a promoter including only the core TATA and, sometimes, the CCAAT elements (Fluhr et al., Science 232:1106-1112, 1986; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986-8990, 1987; Aryan et al., Mol. Gen. Genet. 225:65-71, 1991).

[0083] Promoters are typically comprised of multiple distinct "cis-acting transcriptional regulatory elements," or simply "cis-elements," each of which confers a different aspect of the overall control of gene expression (Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986-8990, 1987; Ellis et al., EMBO J. 6:11-16, 1987; Benfey et al., EMBO J. 9:1677-1684, 1990). "Cis elements" bind trans-acting protein factors that regulate transcription. Some cis elements bind more than one factor, and trans-acting transcription factors may interact with different affinities with more than one cis element (Johnson and McKnight, Ann. Rev. Biochem. 58:799-839, 1989). Plant transcription factors, corresponding cis elements, and analysis of their interaction are discussed, for example, In: Martin, Curr. Opinions Biotech. 7:130-138, 1996; Murai, In: Methods in Plant Biochemistry and Molecular Biology, Dashek, ed., CRC Press, 1997, pp. 397-422; and Methods in Plant Molecular Biology, Maliga et al., eds., Cold Spring Harbor Press, 1995, pp. 233-300.

The promoter sequences of the present invention can contain "cis elements" that confer or modulate gene expression.

[0084] Cis elements can be identified by a number of techniques, including deletion analysis, i.e., deleting one or more nucleotides from the 5' end or internal to a promoter; DNA binding protein analysis using Dnase I footprinting, methylation interference, electrophoresis mobility-shift assays, in. vivo genomic footprinting by ligation-mediated PCR and other conventional assays; or by sequence similarity with known cis element motifs by conventional sequence comparison methods. The fine structure of a cis element can be further studies by mutagenesis (or substitution) of one or more nucleotides of the element or by other conventional methods (see for example, Methods in Plant Biochemistry and Molecular Biology, Dashek, ed., CRC Press, 1997, pp. 397-422; and Methods in Plant Molecular Biology, Maliga et al., eds., Cold Spring Harbor Press, 1995, pp. 233-300).

[0085] Cis elements can be obtained by chemical synthesis or by cloning from promoters that include such elements. Cis elements can also be synthesized with additional flanking sequences that contain useful restriction enzyme sites to facilitate subsequence manipulation. In one embodiment, the promoters are comprised of multiple distinct "cis-elements". In a preferred embodiment sequence regions comprising "cis elements" of the nucleic acid sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 are identified using computer programs including, but not limited to MEME or SIGNALSCAN that are designed specifically to identify cis elements, or domains or motifs within sequences.

[0086] The present invention includes fragments or cis elements of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 or homologues of cis elements known to effect gene regulation that show homology with the nucleic acid sequences of the present invention. Such nucleic acid fragments can include any region of the disclosed sequences. The promoter regions or partial promoter regions of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 can contain at least one regulatory element including, but not limited to "cis elements" or domains that are capable of regulating expression of operably linked DNA sequences, such as in male reproductive tissues.

[0087] Plant promoters can also include promoters produced through the manipulation of known promoters to produce synthetic, chimeric, or hybrid promoters. Such promoters can also combine cis elements from one or more promoters, for example, by adding a heterologous regulatory sequence to an active promoter with its own partial or complete regulatory sequences (Ellis et al., EMBO J. 6:11-16, 1987; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986-8990, 1987; Poulsen and Chua, Mol. Gen. Genet. 214:16-23, 1988; Comai et al., Plant. Mol. Biol. 15:373-381, 1991). Chimeric promoters have also been developed by adding a heterologous regulatory sequence to the 5' upstream region of an inactive, truncated promoter, i.e., a promoter that includes only the core TATA and, optionally, the CCAAT elements (Fluhr et al., Science 232:1106-1112, 1986; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986-8990, 1987; Aryan et al., Mol. Gen. Genet. 225:65-71, 1991).

[0088] Chimeric or hybrid promoters according to the present invention may include at least one known cis element such as elements that are regulated by numerous environmental factors such as light, heat, or stress; elements that are regulated or induced by pathogens or chemicals, and the like. Such elements may either positively or negatively regulate gene expression, depending on the conditions. Examples of cis elements include, but are not limited to oxygen responsive elements (Cowen et al., J. Biol. Chem. 268(36):26904, 1993), light regulatory elements (see for example, Bruce and Quail, Plant Cell 2: 1081, 1990, and Bruce et al., EMBO J. 10:3015, 1991, a cis element reponsive to methyl jasmonate treatment (Beaudoin and Rothstein, Plant Mol. Biol. 33:835, 1997, salicylic acid-responsive elements (Strange et al., Plant J. 11:1315, 1997, heat shock response elements (Pelham et al., Trends Genet. 1:31, 1985, elements responsive to wounding and abiotic stress (Loace et al., Proc. Natl. Acad. Sci. U.S.A. 89:9230, 1992; Mhiri et al., Plant Mol. Biol. 33:257, 1997), cold-responsive elements (Baker et al., Plant Mol. Biol. 24:701, 1994; Jiang et al., Plant Mol. Biol. 30:679, 1996; Nordin et al., Plant Mol. Biol. 21:641, 1993; Zhou et al., J. Biol. Chem. 267:23515, 1992), and drought responsive elements, (Yamaguchi et al., Plant Cell 6:251-264, 1994; Wang et al., Plant Mol. Biol. 28:605, 1995; Bray E. A. Trends in Plant Science 2:48, 1997).

[0089] In another embodiment, the nucleotide sequences as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 includes any length of said nucleotide sequences that is capable of regulating an operably linked DNA sequence. For example, the sequences as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 may be truncated or have portions deleted and still be capable of regulating transcription of an operably linked DNA sequence. In a related embodiment, a cis element of the disclosed sequences may confer a particular specificity such as conferring enhanced expression of operably linked DNA sequences in certain tissues. Consequently, any sequence fragments, portions, or regions of the disclosed sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 can be used as regulatory sequences including but not limited to cis elements or motifs of the disclosed sequences. For example, one or more base pairs may be deleted from the 5' or 3' end of a promoter sequence to produce a "truncated" promoter. One or more base pairs can also be inserted, deleted, or substituted internally to a promoter sequence. Promoters can be constructed such that promoter fragments or elements are operably linked, for example, by placing such a fragment upstream of a minimal promoter. A minimal or basal promoter is a piece of DNA which is capable of recruiting and binding the basal transcription machinery. One example of basal transcription

machinery in eukaryotic cells is the RNA polymerase II complex and its accessory proteins. The enzymatic components of the basal transcription machinery are capable of initiating and elongating transcription of a given gene, utilizing a minimal or basal promoter. That is, there are not added cis-acting sequences in the promoter region that are capable of recruiting and binding transcription factors that modulate transcription, e.g., enhance, repress, render transcription hormone-dependent, etc. Substitutions, deletions, insertions or any combination thereof can be combined to produce a final construct.

[0090] The promoter sequences of the present invention may be modified, for example for expression in other plant systems. In another approach, novel hybrid promoters can be designed or engineered by a number of methods. Many promoters contain upstream sequences which activate, enhance or define the strength and/or specificity of the promoter (Atchison, Ann. Rev. Cell Biol. 4:127, 1988). T-DNA genes, for example contain "TATA" boxes defining the site of transcription initiation and other upstream elements located upstream of the transcription initiation site modulate transcription levels (Gelvin, In: Transgenic Plants (Kung, S.-D. and Us,R., eds, San Diego: Academic Press, pp.49-87, 1988). Another chimeric promoter combined a trimer of the octopine synthase (ocs) activator to the mannopine synthase (mas) activator plus promoter and reported an increase in expression of a reporter gene (Min Ni et al., The Plant Journal 7:661, 1995). The upstream regulatory sequences of the present invention can be used for the construction of such chimeric or hybrid promoters. Methods for construction of variant promoters of the present invention include but are not limited to combining control elements of different promoters or duplicating portions or regions of a promoter (see for example U.S. Pat. No. 5,110,732 and U.S. Pat. No. 5,097,025). Those of skill in the art are familiar with the specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolation of genes, (see for example Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press, 1995; Birren et al., Genome Analysis: volume 1, Analyzing DNA, (1997), volume 2, Detecting Genes, (1998), volume 3, Cloning Systems, (1999) volume 4, Mapping Genomes, (1999), Cold Spring Harbor, N.Y.).

[0091] The design, construction, and use of chimeric or hybrid promoters comprising one or more of cis elements of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, for modulating or regulating the expression of operably linked nucleic acid sequences are also encompassed by the present invention.

[0092] The promoter sequences, fragments, regions or cis elements thereof of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 are capable of transcribing operably linked DNA sequences in multiple tissues and therefore can selectively regulate expression of genes in multiple tissues.

[0093] For a number of agronomic traits, transcription of a gene or genes of interest is desirable in multiple tissues to confer the desired characteristic(s). The availability of suitable promoters that regulate transcription of operably linked genes in selected target tissues of interest is desirable, since it may not be desirable to express a gene in every tissue, but only in certain tissues. For example, if one desires to selectively express a target gene for expression of gene for herbicide tolerance, one may desire expression of the herbicide tolerance gene in vegetative and reproductive tissues. The promoter sequences of the present invention are useful for regulating gene expression in multiple tissues including, but not limited to rapidly growing meristematic tissues, male reproductive tissues (androecium) such as pollen, anthers, and filaments, and female reproductive tissues (gynoecium) such as the stigma, style, and ovaries, leaves, sepals, and petals. The promoters of the present invention therefore have utility for expression of herbicide tolerance genes, for example, where tolerance is desired in multiple tissues and stages of plant development. The promoter sequences of the present invention have utility for regulating transcription of any target gene including but not limited to genes for control of fertility, yield, insect tolerance, fungal tolerance, herbicide tolerance, or any desirable trait of interest. Particularly preferred genes include herbicide tolerance genes or insect tolerance genes.

[0094] In one embodiment, the promoters of the present invention have particular utility for regulating expression of an herbicide tolerance gene where expression of a gene is desired in multiple tissues. For example, the herbicide tolerance gene may confer tolerance to the herbicide glyphosate. Examples of suitable glyphosate tolerance genes include, but are not limited to glyphosate resistant EPSP synthase genes or gene products that degrade glyphosate such as, a glyphosate oxidoreductase and phosphonate N-acetyl transferase. It is important to have a wide variety of choices of 5' regulatory elements for any plant biotechnology strategy in order to have suitable regulatory elements that are most efficient for the expression profile desired.

[0095] In another embodiment, the promoters of the present invention have utility for determining gene function. The function of many genes is unknown and the promoters of the present invention can be used as genetic elements in a construct to allow a phenotypic evaluation of one or more genes expressed in a sense or antisense orientation. The promoters of the present invention can be components in a plant expression construct developed for a high throughput assay where high levels of gene expression in constitutive and reproductive tissues is desired.

[0096] Any plant can be selected for the identification of genes and regulatory sequences. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, Arabidopsis, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine,

pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radisicchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini. Particularly preferred plants for the identification of regulatory sequences are Arabidopsis, corn, wheat, soybean, and cotton.

[0097] The promoter sequences of the present invention were isolated from *Arabidopsis thaliana* plant DNA. In a preferred embodiment, a construct includes the promoter sequences of the present invention operably linked to a transcribable sequence along with suitable terminator and regulatory elements. Such a construct may be transformed into a suitable target plant of interest. Any plant can be used as a suitable host for nucleic acid constructs comprising the promoter sequences of the present invention. Examples of suitable target plants of interest would include, but are not limited to alfalfa, broccoli, cabbage, canola, cauliflower, corn, cotton, cranberry, cucumber, lettuce, pea, poplar, pine, potato, onion, rice, raspberry, soybean, sugarcane, sugarbeet, sunflower, tomato, and wheat.

Promoter Isolation and Modification Methods

[0098] Any number of methods can be used to isolate fragments of the promoter sequences disclosed herein. A PCR-based approach can be used to amplify flanking regions from a genomic library of a plant using publicly available sequence information. A number of methods are known to those of skill in the art to amplify unknown DNA sequences adjacent to a core region of known sequence. Methods include but are not limited to inverse PCR (IPCR), vectorette PCR, Y-shaped PCR and genome walking approaches. For the present invention, the nucleic acid molecules were isolated from Arabidopsis by designing PCR primers based on available sequence information.

[0099] Nucleic acid fragments can also be obtained by other techniques such as by directly synthesizing the fragment by chemical means, as is commonly practiced by using an automated oligonucleotide synthesizer. Fragments can also be obtained by application of nucleic acid reproduction technology, such as the PCR (polymerase chain reaction) technology by recombinant DNA techniques generally known to those of skill in the art of molecular biology. Regarding the amplification of a target nucleic- acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent PCR conditions" refer to conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product.

[0100] Those of skill in the art are aware of methods for the preparation of plant genomic DNA. In one approach, genomic DNA libraries can be prepared from a chosen species by partial digestion with a restriction enzyme and size selecting the DNA fragments within a particular size range. The genomic DNA can be cloned into a suitable construct including but not limited to a bacteriophage, and prepared using a suitable construct such as a bacteriophage using a suitable cloning kit from any number of vendors (see for example Stratagene, La Jolla Calif. or Gibco BRL, Gaithersburg, Md.).

[0101] In another embodiment, the nucleotide sequences of the promoters disclosed herein can be modified. Those skilled in the art can create DNA molecules that have variations in the nucleotide sequence. The nucleotide sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ IDNO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 may be modified or altered to enhance their control characteristics. For example, the sequences may be modified by insertion, deletion or replacement of template sequences in a PCR-based DNA modification approach. "Variant" DNA molecules are DNA molecules containing changes in which one or more nucleotides of a native sequence is deleted, added, and/or substituted, preferably while substantially maintaining promoter function. In the case of a promoter fragment, "variant" DNA can include changes affecting the transcription of a minimal promoter to which it is operably linked. Variant DNA molecules can be produced, for example, by standard DNA mutagenesis techniques or by chemically synthesizing the variant DNA molecule or a portion thereof.

[0102] In addition to their use in modulating gene expression, the promoter sequences of the present invention also have utility as probes or primers in nucleic acid hybridization experiments. The nucleic-acid probes and primers of the present invention can hybridize under stringent conditions to a target DNA sequence. The term "stringent hybridization conditions" is defined as conditions under which a probe or primer hybridizes specifically with a target sequence(s) and not with non-target sequences, as can be determined empirically. The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by the specific hybridization procedure (see for example Sambrook et al., 1989, at 9.52-9.55, Sambrook et al., 1989 at 9.47-9.52, 9.56-9.58; Kanehisa, Nucl. Acids Res. 12:203-213, 1984; and Wetmur and Davidson, J. Mol. Biol. 31:349-370, 1968). Appropriate stringency conditions which promote DNA hybridization are, for example, 6.0×sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2.0×SSC at 50° C., are known to those skilled in the art or can be found in laboratory manuals including but not limited to Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 1989, 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0×SSC at 50° C. to a high stringency of about 0.2×SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C., to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. For example, hybridization using DNA or RNA probes or primers can be performed at 65° C. in 6×SSC, 0.5% SDS, 5×Denhardt's, 100 μg/mL nonspecific DNA (e.g., sonicated salmon sperm DNA) with washing at 0.5×SSC, 0.5% SDS at 65° C., for high stringency.

[0103] A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be

"minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low stringency" conditions. Similarly, the molecules are said to be "complementary" is they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high stringency" conditions. It is contemplated that lower stringency hybridization conditions such as lower hybridization and/or washing temperatures can be used to identify related sequences having a lower degree of sequence similarity if specificity of binding of the probe or primer to target sequence(s) is preserved. Accordingly, the nucleotide sequences of the present invention can be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Detection of DNA segments via hybridization is well-known to those of skill in the art, and thus depending on the application envisioned, one will desire to employ varying hybridization conditions to achieve varying degrees of selectivity of probe towards target sequence and the method of choice will depend on the desired results. Conventional stringency conditions are described in Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989, and by Haymes et al., Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C., 1985.

[0104] In one embodiment of the present invention, the nucleic acid sequences SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, or a fragment, region, cis element, or oligomer of these sequences, are used in hybridization assays of other plant tissues to identify closely related or homologous genes and associated regulatory sequences. These include but are not limited to Southern or northern hybridization assays on any substrate including but not limited to an appropriately prepared plant tissue, cellulose, nylon, or combination filter, chip, or glass slide. Such methodologies are well known in the art and are available in a kit or preparation which can be supplied by commercial vendors.

[0105] A fragment of a nucleic acid as used herein is a portion of the nucleic acid that is less than full-length. For example, for the present invention any length of nucleotide sequence that is less than the disclosed nucleotide sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 is considered to be a fragment. A fragment can also comprise at least a minimum length capable of hybridizing specifically with a native nucleic acid under stringent hybridization conditions as defined above. The length of such a minimal fragment is preferably at least 8 nucleotides, more preferably 15 nucleotides, even more preferably at least 20 nucleotides, and most preferably at least 30 nucleotides of a native nucleic acid sequence.

[0106] A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. "Primers" are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

[0107] Probes and primers are generally 11 nucleotides or more in length, preferably 18 nucleotides or more, more preferably 25 nucleotides, and most preferably 30 nucleotides or more. Such probes and primers hybridize specifically to a target DNA or RNA sequence under high stringency hybridization conditions and hybridize specifically to a target native sequence of another species under lower stringency conditions. Preferably, probes and primers according to the present invention have complete sequence similarity with the native sequence, although probes differing from the native sequence and that retain the ability to hybridize to target native sequences may be designed by conventional methods. Methods for preparing and using probes and primers are described, for example, in Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 (hereinafter, "Sambrook et al., 1989"); Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, N.Y., 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992); and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Primers and probes based on the native promoter sequences disclosed herein can be used to confirm and, if necessary, to modify the disclosed sequences by conventional methods, e.g., by re-cloning and re-sequencing.

Constructs and Expression Constructs

[0108] Native or synthetic nucleic acids according to the present invention can be incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a host cell. In a preferred embodiment, the nucleotide sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 or fragments, variants or derivatives thereof are incorporated into an expression cassette which includes the promoter regions of the present invention operably linked to a genetic component such as a selectable, screenable, or scorable marker gene.

[0109] In another embodiment, the disclosed nucleic acid sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 are operably linked to a genetic component such as a nucleic acid which confers a desirable characteristic associated with plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance. These genetic components such as marker genes or agronomic genes of interest can function in

the identification of a transformed plant cell or plant, or a produce a product of agronomic utility.

[0110] In another embodiment, one genetic component produces a product which serves as a selection device and functions in a regenerable plant tissue to produce a compound which would confer upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to GUS (coding sequence for beta-glucuronidase), GFP (coding sequence for green fluorescent protein), LUX (coding gene for luciferase), antibiotic resistance marker genes, or herbicide tolerance genes. Examples of transposons and associated antibiotic resistance genes include the transposons Tns (bla), Tn5 (nptII), Tn7 (dhfr), penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline.

[0111] Characteristics useful for selectable markers in plants have been outlined in a report on the use of microorganisms (Advisory Committee on Novel Foods and Processes, July 1994). These include stringent selection with minimum number of nontransformed tissues, large numbers of independent transformation events with no significant interference with the regeneration, application to a large number of species, and availability of an assay to score the tissues for presence of the marker.

[0112] A number of selectable marker genes are known in the art and several antibiotic resistance markers satisfy these criteria, including those resistant to kanarnycin (nptII), hygromycin B (aph IV) and gentamycin (aac3 and aacC4). Useful dominant selectable marker genes include genes encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin); and herbicide resistance genes (e.g., phosphinothricin acetyltransferase). A useful strategy for selection of transformants for herbicide resistance is described, e.g., in Vasil, Cell Culture and Somatic Cell Genetics of Plants, Vols. I-III, Laboratory Procedures and Their Applications Academic Press, N.Y., 1984. Particularly preferred selectable marker genes for use in the present invention would genes which confer resistance to compounds such as antibiotics like kanamycin, and herbicides like glyphosate (Della-Cioppa et al., Bio/Technology 5(6), 1987, U.S. Pat. No. 5,463,175, U.S. Pat. No. 5,633,435). Other selection devices can also be implemented and would still fall within the scope of the present invention.

[0113] For the practice of the present invention, conventional compositions and methods for preparing and using DNA constructs and host cells are employed, as discussed, inter alia, in Sambrook et al., 1989. In a preferred embodiment, the host cell is a plant cell. A number of DNA constructs suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; and R.R.D. Croy Plant Molecular Biology LabFax, BIOS Scientific Publishers, 1993. Plant expression constructs can include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences. They can also include a selectable marker as described to select for host cells containing the expression construct. Such plant expression constructs also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and a polyadenylation signal. Other sequences of bacterial origin are also included to allow the construct to be cloned in a bacterial host. The construct will also typically contain a broad host range prokaryotic origin of replication. In a particularly preferred embodiment, the host cell is a plant cell and the plant expression construct comprises a promoter region as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; an operably linked transcribable sequence; and a transcription termination sequence. Other regulatory sequences envisioned as genetic components in an expression construct include but is not limited to non-translated leader sequence which can be coupled with the promoter. In a particularly preferred embodiment, the host cell is a plant cell and the plant expression construct comprises a promoter region as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; an operably linked transcribable sequence, and a transcription termination sequence. Plant expression constructs also can comprise additional sequences including but not limited to polylinker sequences that contain restriction enzyme sites that are useful for cloning purposes.

Genetic Elements in Plant Expression Constructs

[0114] Plant expression constructs may include more than one expressible gene sequence, each operably linked to a different promoter. A number of promoters have utility for plant gene expression for any gene of interest including but not limited to selectable markers, scorable markers, genes for pest tolerance, disease tolerance, nutritional enhancements and any other gene of agronomic interest. Examples of constitutive promoters useful for plant gene expression include but are not limited to, the cauliflower mosaic virus (CaMV) P-35S promoter, which confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., Nature 313:810, 1985), including monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220:389, 1990); a tandemly duplicated version of the CaMV 35S promoter, the enhanced 35S promoter (P-e35S) the nopaline synthase promoter (An et al., Plant Physiol. 88:547, 1988), the octopine synthase promoter (Fromm et al., Plant Cell 1:977, 1989); and the figwort mosaic virus (P-FMV) promoter as described in U.S. Pat. No. 5,378,619 and an enhanced version of the FMV promoter (P-eFMV) where the promoter sequence of P-FMV is duplicated in tandem, the cauliflower mosaic virus 19S promoter, a sugarcane bacilliform virus promoter, a commelina yellow mottle virus promoter, and other plant DNA virus promoters known to express in plant cells.

[0115] A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals can be used for expression of an

operably linked gene in plant cells, including promoters regulated by (1) heat (Callis et al., Plant Physiol. 88:965, 1988), (2) light (e.g., pea rbcS-3A promoter, Kuhlemeier et al., Plant Cell 1:471, 1989; maize rbcS promoter, Schaffner and Sheen, Plant Cell 3:997, 1991; or chlorophyll a/b-binding protein promoter, Simpson et al., EMBO J. 4:2723, 1985), (3) hormones, such as abscisic acid (Marcotte et al., Plant Cell 1:969, 1989), (4) wounding (e.g., wuni, Siebertz et al., Plant Cell 1:961, 1989); or (5) chemicals such as methyl jasmonate, salicylic acid, or Safener. It may also be advantageous to employ (6) organ-specific promoters (e.g., Roshal et al., EMBO J. 6:1155, 1987; Schernthaner et al., EMBO J. 7:1249, 1988; Bustos et al., Plant Cell 1:839, 1989). The promoters of the present invention are plant promoters that are capable of transcribing operably linked DNA sequences in rapidly growing meristematic tissue and reproductive tissues and can be operably linked to any gene of interest in an expression construct.

[0116] Plant expression constructs can include RNA processing signals, e.g., introns, which may be positioned upstream or downstream of a polypeptide-encoding sequence in the transgene. In addition, the expression constructs may include additional regulatory sequences from the 3'-untranslated region of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. USA 84:744 (1987); An et al., Plant Cell 1:115 (1989), e.g., a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions. 5' non-translated regions of a MRNA can play an important role in translation initiation and can also be a genetic component in a plant expression construct. For example, non-translated 5' leader sequences derived from heat shock protein genes have been demonstrated to enhance gene expression in plants (see, for example U.S. Pat. No. 5,362,865). These additional upstream and downstream regulatory sequences may be derived from a source that is native or heterologous with respect to the other elements present on the expression construct.

[0117] The promoter sequences of the present invention are used to control gene expression in plant cells. The disclosed promoter sequences are genetic components that are part of constructs used in plant transformation. The promoter sequences of the present invention can be used with any suitable plant transformation plasmid or construct containing a selectable or screenable marker and associated regulatory elements, as described, along with one or more nucleic acids expressed in a manner sufficient to confer a particular desirable trait. Examples of suitable structural genes of agronomic interest envisioned by the present invention would include but are not limited to one or more genes for insect tolerance, such as a *Bacillus thuringiensis* (B.t.) gene, pest tolerance such as genes for fungal disease control, herbicide tolerance such as genes conferring glyphosate tolerance, and genes for quality improvements such as yield, nutritional enhancements, environmental or stress tolerances, or any desirable changes in plant physiology, growth, development, morphology or plant product(s). For example, structural genes would include any gene that confers insect tolerance including but not limited to a Bacillus insect control protein gene as described in WO 9931248, herein incorporated by reference in its entirety, U.S. Pat. No. 5,689,052, herein incorporated by reference in its entirety, U.S. Pat. Nos. 5,500,365 and 5,880275, herein incorporated by reference it their entirety. In another embodiment, the structural gene can confer tolerance to the herbicide glyphosate as conferred by genes including, but not limited to Agrobacterium strain CP4 glyphosate resistant EPSPS gene (aroA:CP4) as described in U.S. Pat. No. 5,633,435, herein incorporated by reference in its entirety, or glyphosate oxidoreductase gene (GOX) as described in U.S. Pat. No. 5,463,175, herein incorporated by reference in its entirety.

[0118] Alternatively, the DNA coding sequences can effect these phenotypes by encoding a non-translatable RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via antisense- or cosuppression-mediated mechanisms (see, for example, Bird et al., Biotech. Gen. Engin. Rev. 9:207,1991). The RNA could also be a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave a desired endogenous MRNA product (see for example, Gibson and Shillitoe, Mol. Biotech. 7:125,1997). Thus, any gene which produces a protein or mRNA which expresses a phenotype or morphology change of interest is useful for the practice of the present invention.

[0119] In addition to regulatory elements or sequences located upstream (5') or within a DNA sequence, there are downstream (3') sequences that affect gene expression. Thus, the term regulatory sequence as used herein refers to any nucleotide sequence located upstream, within, or downstream to a DNA sequence which controls, mediates, or affects expression of a gene product in conjunction with the protein synthetic apparatus of the cell.

[0120] Those of skill in the art are aware of the constructs suitable for plant transformation. The promoter sequences of the present invention are preferably incorporated into an expression construct using screenable or scorable markers as described and tested in transient analyses to provide an indication of gene expression in transformed plants. Methods of testing gene expression in transient assays are known to those of skill in the art. Transient expression of marker genes has been reported using a variety of plants, tissues and DNA delivery systems. For example, types of transient analyses can include but are not limited to direct gene delivery via electroporation or particle bombardment of tissues in any transient plant assay using any plant species of interest. Such transient systems would include but are not limited to protoplasts from suspension cultures in wheat (Zhou et al., Plant Cell Reports 12:612. 1993), electroporation of leaf protoplasts of wheat (Sethi et al., J. Crop Sci. 52: 152, 1983); electroporation of protoplast prepared from corn tissue (Sheen, J. The Plant Cell 3: 225, 1991), or particle bombardment of specific tissues of interest. The present invention encompasses the use of any transient expression system to evaluate regulatory sequences operatively linked to selected reporter genes, marker genes or agronomic genes of interest. Examples of plant tissues envisioned to test in transients via an appropriate delivery system would include but are not limited to leaf base tissues, callus, cotyledons, roots, endosperm, embryos, floral tissue, pollen, and epidermal tissue.

[0121] Any scorable or screenable marker can be used in a transient assay. Preferred marker genes for transient analyses of the promoters or 5' regulatory sequences of the present invention include a P-glucuronidase (GUS) gene or a green fluorescent protein (GFP) gene. The expression constructs containing the 5' regulatory sequences operably linked to a marker gene are delivered to the tissues and the tissues are

analyzed by the appropriate mechanism, depending on the marker. The quantitative or qualitative analyses are used as a tool to evaluate the potential expression profile of the 5' regulatory sequences when operatively linked to genes of agronomic interest in stable plants. Ultimately, the 5' regulatory sequences of the present invention are directly incorporated into suitable plant transformation expression constructs comprising the 5' regulatory sequences operatively linked to a transcribable DNA sequence interest, transformed into plants and the stably transformed plants and progeny thereof analyzed for the desired expression profile conferred by the 5' regulatory sequences.

[0122] Suitable expression constructs for introducing exogenous DNA into plant cells would include but are not limited to disarmed Ti-plasmids for Agrobacterium-mediated methods. These constructs can contain a resistance marker, 1-2 T-DNA borders, and origins of replication for *E. coli* and Agrobacterium along with one or more genes of interest and associated regulatory regions. Those of skill in the art are aware that for Agrobacterium-mediated approaches a number of strains and methods are available. Such strains would include but are not limited to Agrobacterium strains C58, LBA4404, EHA101 and EHA105. Particularly preferred strains are *Agrobacterium tumefaciens* strains.

[0123] Exemplary nucleic acids which may be introduced by the methods encompassed by the present invention include, for example, DNA sequences or genes from another species, or even genes or sequences which originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. However, the term "exogenous" is also intended to refer to genes that are not normally present in the cell being transformed, or perhaps simply not present in the form, structure, etc., as found in the transforming DNA segment or gene, or genes which are normally present and that one desires to express in a manner that differs from the natural expression pattern, e.g., to over-express. Thus, the term "exogenous" gene or DNA is intended to refer to any gene or DNA segment that is introduced into a recipient cell, regardless of whether a similar gene may already be present in such a cell. The type of DNA included in the exogenous DNA can include DNA which is already present in the plant cell, DNA from another plant, DNA from a different organism, or a DNA generated externally, such as a DNA sequence containing an antisense message of a gene, or a DNA sequence encoding a synthetic or modified version of a gene.

[0124] The plant transformation constructs containing the promoter sequences of the present invention may be introduced into plants by any plant transformation method. Several methods are available for introducing DNA sequences into plant cells and are well known in the art. Suitable methods include but are not limited to bacterial infection (e.g., with Agrobacterium as described above), binary bacterial artificial chromosome constructs, direct delivery of DNA (e.g. via PEG-mediated transformation, desiccation/inhibition-mediated DNA uptake, electroporation, agitation with silicon carbide fibers), and acceleration of DNA coated particles (reviewed in Potrykus, Ann. Rev. Plant Physiol. Plant Mol. Biol., 42: 205, 1991).

[0125] Methods for specifically transforming dicots primarily use *Agrobacterium tumefaciens*. For example, transgenic plants reported include but are not limited to cotton (U.S. Pat. No. 5,004,863; U.S. Pat. No. 5,159,135; U.S. Pat. No. 5,518,908; WO 97/43430), soybean (U.S. Pat. No. 5,569,834; U.S. Pat. No. 5,416,011; McCabe et al., Bio/Technology, 6:923, 1988; Christou et al., Plant Physiol., 87:671, 1988); Brassica (U.S. Pat. No. 5,463,174), and peanut (Cheng et al., Plant Cell Rep., 15: 653, 1996).

[0126] Similar methods have been reported in the transformation of monocots. Transformation and plant regeneration using these methods have been described for a number of crops including but not limited to asparagus (*Asparagus officinalis;* Bytebier et al., Proc. Natl. Acad. Sci. U.S.A., 84: 5345, 1987); barley (*Hordeum vulgarae;* Wan and Lemaux, Plant Physiol., 104: 37, 1994); maize (*Zea mays;* Rhodes, C. A., et al., Science, 240: 204, 1988; Gordon-Kamm, et al., Plant Cell, 2: 603, 1990; Fromm, et al., Bio/Technology, 8: 833, 1990; Koziel, et al., Bio/Technology, 11: 194, 1993); oats (*Avena sativa;* Somers, et al., Bio/Technology, 10: 1589, 1992); orchardgrass (*Dactylis glomerata;* Horn, et al., Plant Cell Rep., 7: 469, 1988); rice (*Oryza sativa,* including indica and japonica varieties, Toriyama, et al., Bio/Technology, 6: 10, 1988; Zhang, et al., Plant Cell Rep., 7: 379, 1988; Luo and Wu, Plant Mol. Biol. Rep., 6: 165, 1988; Zhang and Wu, Theor. Appl. Genet., 76: 835, 1988; Christou, et al., Bio/Technology, 9: 957, 1991); sorghum (*Sorghum bicolor;* Casas, A.M., et al., Proc. Natl. Acad. Sci. U.S.A., 90: 11212; 1993); sugar cane (*Saccharum spp.;* Bower and Birch, Plant J., 2: 409, 1992); tall fescue (*Festuca arundinacea;* Wang, Z. Y. et al., Bio/Technology, 10: 691, 1992); turfgrass (*Agrostis palustris;* Zhong et al., Plant Cell Rep., 13: 1, 1993); wheat (*Triticum aestivum;* Vasil et al., Bio/Technology, 10: 667, 1992; Weeks T., et al., Plant Physiol., 102: 1077, 1993; Becker, et al., Plant, J. 5: 299, 1994), and alfalfa (Masoud, S. A., et al., Transgen. Res., 5: 313, 1996). It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

Plant Analysis Methods

[0127] The transformed plants are analyzed for the presence of the genes of interest and the expression level and/or profile conferred by the promoter sequences of the present invention. Those of skill in the art are aware of the numerous methods available for the analysis of transformed plants. A variety of methods are used to assess gene expression and determine if the introduced gene(s) is integrated, functioning properly, and inherited as expected. For the present invention the promoters can be evaluated by determining the expression levels of genes to which the promoters are operatively linked. A preliminary assessment of promoter function can be determined by a transient assay method using reporter genes, but a more definitive promoter assessment can be determined from the analysis of stable plants. Methods for plant analysis include but are not limited to Southern blots or northern blots, PCR-based approaches, biochemical analyses, phenotypic screening methods, field evaluations, and immunodiagnostic assays.

[0128] The methods of the present invention including but not limited to PCR technologies, genomic DNA isolation, expression construct construction, transient assays, and plant transformation methods are well known to those of skill in the art and are carried out using standard techniques or modifications thereof.

Glyphosate Spray Tests

[0129] In one embodiment a greenhouse or field evaluation for glyphosate tolerance is conducted. The term "glyphosate" is used herein to refer collectively to the parent herbicide N-phosphonomethylglycine (otherwise known as glyphosate acid), to a salt or ester thereof, or to a compound which is converted to N-phosphonomethylglycine in plant tissues or which otherwise provides N-phosphonomethylglycine in ionic form (otherwise known as glyphosate ion). Illustratively, water-soluble glyphosate salts useful herein are disclosed in U.S. Pat. Nos. 3,799,758 and 4,405,531 to Franz, the disclosure of which is incorporated herein by reference. Glyphosate salts that can be used according to the present invention include but are not restricted to alkali metal, for example sodium and potassium, salts; ammonium salt; C_{1-16} alkylammonium, for example dimethylammonium and isopropylamrnonium, salts; C_{1-16} alkanolammonium, for example monoethanolammonium, salt; C_{1-16} alkylsulfonium, for example trimethylsulfonium, salts; mixtures thereof and the like. The glyphosate acid molecule has three acid sites having different pKa values; accordingly mono-, di- and tribasic salts, or any mixture thereof, or salts of any intermediate level of neutralization, can be used.

[0130] Glyphosate salts are commercially significant in part because they are water-soluble. Many ammonium, alkylammonium, alkanolammonium, alkylsulfonium and alkali metal salts are highly water-soluble, allowing for formulation as highly concentrated aqueous solutions which can be diluted in water at the point of use.

[0131] Such concentrated aqueous solutions can contain about 50 to about 500 grams per liter of glyphosate, expressed as acid equivalent (g a.e./l). Higher glyphosate concentrations, for example about 300 to about 500 g a.e./l, are preferred.

[0132] Glyphosate salts are alternatively formulated as water-soluble or water-dispersible compositions, in the form for example of powders, granules, pellets or tablets. Such compositions are often known as dry formulations, although the term "dry" should not be understood in this context to imply the complete absence of water. Typically, dry formulations contain less than about 5% by weight of water, for example about 0.5% to about 2% by weight of water. Such formulations are intended for dissolution or dispersion in water at the point of use.

[0133] Contemplated dry glyphosate formulations can contain about 5% to about 80% by weight of glyphosate, expressed as acid equivalent (% a.e.). Higher glyphosate concentrations within the above range, for example about 50% to about 80% a.e., are preferred. Especially useful salts of glyphosate for making dry formulations are sodium and ammonium salts.

[0134] Plant treatment compositions and liquid and dry concentrate compositions of the invention can optionally contain one or more desired excipient ingredients. Especially useful excipient ingredients for glyphosate compositions are surfactants, which assist in retention of aqueous spray solutions on the relatively hydrophobic surfaces of plant leaves, as well as helping the glyphosate to penetrate the waxy outer layer (cuticle) of the leaf and thereby contact living tissues within the leaf. Surfactants can perform other useful functions as well.

[0135] There is no restriction in the type or chemical class of surfactant that can be used in glyphosate compositions of the invention. Nonionic, anionic, cationic and amphoteric types, or combinations of more than one of these types, are all useful in particular situations. However, it is generally preferred that at least one of the surfactants, if any, present should be other than anionic; i.e., at least one of the surfactants should be nonionic, cationic or amphoteric.

[0136] Many surfactants useful herein have a chemical structure that comprises one or more moieties each consisting of a single C_{2-4} alkylene oxide unit or a polymerized or copolymerized chain of C_{2-4} alkylene oxide units. Such surfactants are referred to as polyoxyalkylene surfactants and include nonionic, anionic, cationic and amphoteric types. Polyoxyalkylene surfactants useful in presently contemplated compositions contain about 2 to about 100 C_{2-4} alkylene oxide units. In preferred polyoxyalkylene surfactants the alkylene oxide units form one or more chains of either ethylene oxide or copolymerized ethylene oxide and propylene oxide, each chain of alkylene oxide units having a terminal hydrido group or a C_{1-4} alkyl or C_{1-4} alkanoyl end-cap.

[0137] Hydrophobic moieties of surfactants useful in compositions of the invention can be essentially hydrocarbon based, in which case the hydrophobic moieties are typically C_{8-24}, preferably C_{12-18}, alkyl, alkenyl, alkylaryl, alkanoyl or alkenoyl chains. These chains can be linear or branched. Alternatively, the hydrophobic moieties can contain silicon atoms, for example in the form of siloxane groups such as heptamethyltrisiloxane groups, or fluorine atoms, for example as partially-fluorinated alkyl or perfluoroalkyl chains.

[0138] Among nonionic surfactants, especially preferred classes include polyoxyethylene alkyl, alkenyl or alkylaryl ethers, such as ethoxylated primary or secondary alcohols or alkylphenols, polyoxyethylene alkyl or alkenyl esters, such as ethoxylated fatty acids, polyoxyethylene sorbitan alkyl esters, glyceryl alkyl esters, sucrose esters, alkyl polyglycosides, and the like. Representative specific examples of such nonionic surfactants include polyoxyethylene (9) nonylphenol, Neodol™ 25-7 of Shell (a polyoxyethylene (7) C_{12-15} linear primary alcohol), Tergitol™ 15-S-9 of Union Carbide (a polyoxyethylene (9) C_{12-15} secondary alcohol), Tween™ 20 of ICI (a polyoxyethylene (20) sorbitan monolaurate) and Agrimul™ PG-2069 of Henkel (a C_{9-11} alkyl polyglucoside).

[0139] Among cationic surfactants, especially preferred classes include polyoxyethylene tertiary alkylamines or alkenylamines, such as ethoxylated fatty amines, quaternary ammonium surfactants, polyoxyethylene alkyletheramines, and the like. Representative specific examples of such cationic surfactants include polyoxyethylene (5) cocoamine, polyoxyethylene (15) tallowamine, distearyldimethylammonium chloride, cetyltrimethylammonium bromide, methyl bis(2-hydroxyethyl)cocoammonium chloride, N-dodecylpyridine chloride and polyoxypropylene (8) ethoxytrimethylammonium chloride. Particularly preferred polyoxyethylene alkyletheramines are those disclosed in PCT Publication No. WO 96/32839. Many cationic quaternary ammonium surfactants of diverse structures are known in the art to be useful in combination with glyphosate and can be used in

compositions contemplated herein; such quaternary ammonium surfactants have the formula

$(NR^aR^bR^cR^d)_m{\wedge}n$

[0140] where A is a suitable anion such as chloride, bromide, iodide, acetate, sulfate or phosphate, m and n are integers such that the positive electrical charges on cations ($NR^aR^bR^cR^d$) balance the negative electrical charges on anions A, and options for R^a, R^b, R^c and R^d include, without limitation:

[0141] (i) R^a is benzyl or C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, and R^b, R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

[0142] (ii) R^a and R^b are independently C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, and R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

[0143] (iii) R^a is C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, R^b is a polyoxyalkylene chain having about 2 to about 100 C_{2-4} alkylene oxide units, preferably ethylene oxide units, and R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

[0144] (iv) R^a is C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, R^b and R^c are polyoxyalkylene chains having in total about 2 to about 100 C_{2-4} alkylene oxide units, preferably ethylene oxide units, and R^d is C_{1-4} alkyl, preferably methyl; or

[0145] (v) R^a is a polyoxyalkylene chain having about 2 to about 100 C_{2-4} alkylene oxide units in which C_{3-4} alkylene oxide units, preferably propylene oxide units, predominate and R^b, R^c and R^d are independently C_{1-4} alkyl, preferably methyl or ethyl.

[0146] Particularly preferred quaternary ammonium surfactants of this type are those disclosed in U.S. Pat. No. 5,464,807 to Claude et al.

[0147] In one embodiment, the anion A associated with such a quaternary ammonium surfactant can be a glyphosate anion.

[0148] Among amphoteric surfactants, including as is customary in the art surfactants more correctly described as zwitterionic, especially preferred classes include polyoxyethylene alkylamine oxides, alkylbetaines, alkyl-substituted amino acids and the like Representative examples of such amphoteric surfactants include dodecyldimethylamine oxide, polyoxyethylene (2) cocoamine oxide and stearyldimethylbetaine.

[0149] Standard reference sources from which one of skill in the art can select suitable surfactants, without limitation to the above mentioned classes, include *Handbook of Industrial Surfactants,* Second Edition (1997) published by Gower, *McCutcheon's Emulsifiers and Detergents,* North American and International Editions (1997) published by MC Publishing Company, and *International Cosmetic Ingredient Dictionary,* Sixth Edition (1995) Volumes 1 and 2, published by the Cosmetic, Toiletry and Fragrance Association.

[0150] Other optional components of compositions of the invention include agents to modify color, viscosity, gelling properties, freezing point, hygroscopicity, caking behavior, dissolution rate, dispersibility, or other formulation characteristics.

[0151] Examples of commercial formulations of glyphosate include, without restriction, those sold by Monsanto Company as ROUNDUP®, ROUNDUP® ULTRA, ROUNDUP® CT, ROUNDUP® EXTRA, ROUNDUP® BIACTIVE, ROUNDUP® BIOFORCE, RODEO®), POLARIS(®, SPARK® and ACCORD® herbicides, all of which contain glyphosate as its isopropylammonium salt; those sold by Monsanto Company as ROUNDUP® DRY and RIVAL® herbicides, which contain glyphosate as its ammonium salt; that sold by Monsanto Company as ROUNDUP® GEOFORCE, which contains glyphosate as its sodium salt; and that sold by Zeneca Limited as TOUCH-DOWN® herbicide, which contains glyphosate as its trimethylsulfonium salt.

[0152] The selection of application rates for a glyphosate formulation that are biologically effective is within the skill of the ordinary agricultural technician. One of skill in the art will likewise recognize that individual plant conditions, weather conditions and growing conditions can affect the results achieved in practicing the process of the present invention. Over two decades of glyphosate use and published studies relating to such use have provided abundant information from which a weed control practitioner can select glyphosate application rates that are herbicidally effective on particular species at particular growth stages in particular environmental conditions.

[0153] A process of the present invention is applicable to any and all plant species on which glyphosate is biologically effective as a herbicide or plant growth regulator. This encompasses a very wide variety of plant species worldwide. Likewise, compositions of the invention can be applied to any and all plant species on which glyphosate is biologically effective.

[0154] In one embodiment, a glyphosate-containing herbicide is applied to the plant comprising the DNA constructs of the present invention, and the plants are evaluated for tolerance to the glyphosate herbicide. Any formulation of glyphosate can be used for testing plants comprising the DNA constructs of the present invention. For example, a glyphosate composition such as Roundup Ultra™ can be used. The testing parameters for an evaluation of the glyphosate tolerance of the plant will vary depending on a number of factors. Factors would include, but are not limited to the type of glyphosate formulation, the concentration and amount of glyphosate used in the formulation, the type of plant, the plant developmental stage during the time of the application, environmental conditions, the application method, and the number of times a particular formulation is applied. For example, plants can be tested in a greenhouse environment using a spray application method. The testing range using Roundup Ultra™ can include, but is not limited to 8 oz/acre to 256 oz/acre. The preferred commercially effective range can be from 16 oz/acre to 64 oz/acre of Roundup Ultra™, depending on the crop and stage of plant development. A crop can be sprayed with at least one application of a glyphosate formulation. For testing in cotton an application of 32 oz/acre at the 3-leaf stage may be followed by additional applications at later stages in development. For wheat an application of 32 oz/acre of Roundup Ultra™ at the 3-5 leaf stage can be used and may be followed with a pre- or post-harvest application, depending on the type of wheat to be tested. The test parameters can be optimized for each crop in order to find the particular plant

comprising the constructs of the present invention that confers the desired commercially effective glyphosate tolerance level.

[0155] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

EXAMPLES

Example 1

[0156] The plasmid constructs used are either pUC cloning constructs or double border plant transformation constructs containing an *E. coli* origin of replication such as ori322, a broad host range origin of replication such as oriV or oriRi, and a coding region for a selectable marker such as Spc/Str that encodes for Tn7 aminoglycoside adenyltransferase (aadA) confers resistance to spectinomycin or streptomycin, or a gentamicin (Gm, Gent) selectable marker. For plant transformation, the host bacterial strain was *Agrobacterium tumefaciens* ABI or LBA4404.

[0157] The genetic elements are described as follows: P-e35S is the 35S RNA from CaMV containing a duplication of the −90–300 region as described in U.S. Pat. No. 5,424,200 herein incorporated by reference in its entirety; P-FMV is the 34S promoter from Figwort Mosaic Virus as described in U.S. Pat. No. 5,378,619 herein incorporated by reference in its entirety; P-eFMV is a derivative of the FMV promoter containing a duplicated enhancer region of the FMV promoter; CTP2 is the transit peptide region of Arabidopsis EPSP synthase as described in U.S. Pat. No. 5,633,435; aroA:CP4syn (aroA:CP4) is the coding region for CP4 EPSP (synthetic sequence) as described in U.S. Pat. No. 5,633,435 or further modified for expression in plants based on codon usage of particular plant species; E9 3' is the 3' end of an isolate of the pea RbcS gene that functions as a polyadenylation signal; nos is the 3' end of the nopaline synthase gene that functions as a polyadenylation signal; Hsp70 is the non-translated leader sequence from *Petunia hybrida* as described in U.S. Pat. No. 5,362,865 herein incorporated by reference in its entirety; GUS is the beta-glucuronidase coding sequence from *E. coli* (Jefferson, R. A. *Proc. Natl. Acad. Sci.* U.S.A., 83:8447-8451, 1987); the right border (RB) and left borders (LB) are from the Ti plasmid of *Agrobacterium tumefaciens* octopine and nopaline strains. The P-AtAct2 is the promoter from the *Arabidopsis thaliana* actin 2 gene; AtAct2i is the intron in the 5' untranslated region (UTR) of the *Arabidopsis thaliana* actin 2 gene; P-AtAct8 is the promoter from the *Arabidopsis thaliana* actin 8 gene; AtAct2i is the intron in the 5' UTR of the *Arabidopsis thaliana* actin 8 gene; P-AtAct11 is the promoter from the *Arabidopsis thaliana* actin 11 gene; AtAct11i is the intron in the 5' UTR of the *Arabidopsis thaliana* actin 11 gene; P-AtAct1a is the promoter from the *Arabidopsis thaliana* actin 1a gene, L-AtAct1a is the untranslated leader and I-AtAct1a is the intron from the genomic DNA of the actin 1a gene; P-AtAct1b is the promoter from the *Arabidopsis thaliana* actin 1b gene, L-AtAct1b is the untranslated leader and I-AtAct1b is the intron from the genomic DNA of the actin 1b gene; P-AtAct3 is the promoter from the *Arabidopsis thaliana* actin 3 gene, L-AtAct3 is the untranslated leader and I-AtAct3 is the intron from the genomic DNA of the actin 3 gene; P-AtAct7 is the promoter from the *Arabidopsis thaliana* actin 7 gene, L-AtAct7 is the untranslated leader and I-AtAct7 is the intron from the genomic DNA of the actin 7 gene; P-AtAct12 is the promoter from the *Arabidopsis thaliana* actin 12 gene, L-AtAct12 is the untranslated leader and I-AtAct12 is the intron from the genomic DNA of the actin 12 gene; P-AtEF1α (P-AtEF1 or EF1α) is the promoter from the *Arabidopsis thaliana* elongation factor gene 1α, AtEF1α-i (AtEF1α-i) is the intron in the 5' UTR of the *Arabidopsis thaliana* elongation factor gene 1α.

[0158] FIGS. 1-18 provide examples of plant transformation constructs that contain one to three plant expression cassettes. Multiple combinations of plant expression cassettes comprising the promoter and genetic elements of the present invention can be constructed and tested in crops plants by those skilled in the art of plant molecular biology without undue experimentation. The constructs illustrated in the Figures are not to be construed as the only constructs that can be assembled, but serve only as examples to those skilled in the art. **FIG. 1** (pCGN8086) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct8) operably linked to a gene of interest (CTP2-aroA:CP4syn). **FIG. 2** (pMON45325) provides an example of a plant transformation construct containing two expression cassettes comprising at least one promoter of the present invention (P-AtAct11) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). **FIG. 3** (pMON45331) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtEF 1 plus intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). **FIG. 4** (pMON45332) provides an example of a plant transformation construct containing two expression cassettes comprising at least one promoter of the present invention (P-AtEF1 plus intron) operably linked to at least one gene of interest (CTP2-aroA CP4syn). **FIG. 5** (pMON9190) provides an example of a plant transformation construct containing three expression cassettes wherein at least two promoters of the present invention (P-ATEF1 plus intron, AtEF1α-i; P-AtAct2 plus intron, AtAct2i) are operably linked to at least one gene of interest (CTP2-aroA:CP4syn) and the P-eFMV promoter operably lined to CTP2-aroA:CP4syn. **FIG. 6** (pMON9153) plant expression cassettes are identical to those illustrated in **FIG. 4** (pMON45332), this plasmid map is illustrated for the purpose of identification of the expression cassettes for data shown on plant phenotype in the data tables shown in the specification. **FIG. 7** (pCGN8099) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-eFMV-AtEF1α and P-e35S-AtAct8, driving transcription of the gene of interest (aroA:CP4syn). **FIG. 8** (pCGN8088) provides an example of a plant transformation construct containing two expression cassettes comprising

one promoter of the present invention, P-AtAct8 plus intron, AtAct8i, and the P-eFMV promoter driving expression of a gene of interest (aroA:CP4syn). **FIG. 9** (pCGN8068) provides an example of a plant transformation construct containing two expression cassettes comprising one promoter of the present invention, P-AtAct2 plus intron, AtAct2i, and the P-eFMV promoter driving expression of a gene of interest (aroA:CP4syn). **FIG. 10** (pCGN8096) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-eFMV/-AtAct11 and P-e35S-AtAct2, driving transcription of the gene of interest (aroA:CP4syn). **FIG. 11** (pCGN9151) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-eFMV-AtEF1α and P-e35S-AtAct2, driving transcription of the gene of interest (aroA:CP4syn). **FIG. 12** (pMON10156) provides an example of a plant transformation construct containing one expression cassette comprising the P-eFMV promoter driving expression of the aroA:CP4syn gene of interest, this vector is used for comparative purposes with the promoter sequences of the present invention. **FIG. 13** (pMON52059) provides an example of a plant transformation construct containing one expression cassette comprising a hybrid promoter (P-eFMV-AtEF1α) driving the expression of the gene of interest (aroA:CP4syn). **FIG. 14** (pMON54952) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct1a plus AtAct1a intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). **FIG. 15** (pMON54953) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct1b plus AtAct1b intron) operably linked to at least one gene of interest (CTP2-aroA-CP4syn). **FIG. 16** (pMON54954) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct3 plus AtAct3 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). **FIG. 17** (pMON54955) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct7 plus AtAct7 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). **FIG. 18** (pMON54956) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct12 plus AtAct12 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn).

Example 2

[0159] The cloning constructs and GUS constructs are listed in Table 1. The Arabidopsis actin 2 promoter and intron (Genbank accession number U41998 as described in An et al., Plant J. 10: 107-121, 1996) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template (Rogers and Bendich, Plant Mol. Biol. 5:69, 1998) using SEQ ID NO:1 (forward primer) and SEQ ID NO:2 (reverse primer) in a reaction as follows: 0.5 μg template DNA, 25 pmole of each primer, taq polymerase (BMB, Indianapolis, Ind.) using wax beads for "hot start" PCR. The PCR thermocycler conditions were as follows: 94° C. for one minute; 30 cycles of: 92° C. for 40 seconds, 55° C. for one minute, 72° C. for one minute and 30 seconds; and a five minute 72° C. extension. The PCR reaction was purified using GeneClean II (Bio101 Inc., Vista, Calif.), digested with HindIII and NcoI, and ligated into construct pMON26149 (Table 1) digested with HindIII and NcoI. The promoter clone was sequence verified and the resulting construct was designated pMON26170 (Table 1).

TABLE 1

Cloning Constructs and GUS Constructs containing Arabidopis Actin and EF1 promoter sequences

Construct	Description	Promoter*/Gene/3'
pMON26149	cloning construct	
pMON26170	plant expression construct	Act2/GUS/nos
pMON26171	plant expression construct	Act8/GUS/nos
pMON8677	cloning construct	
pMON48407	plant expression construct	Act11/GUS/nos
pMON26152	cloning construct	
pMON26177	plant expression construct	EF1/GUS/nos
pMON11750	plant expression construct	e35S/GUS/nos
pMON15737	plant expression construct	FMV/GUS/nos

*the actin and elongation factor promoter sequences also contain the intron sequence from the 5' UTR of the corresponding gene.

Example 3

[0160] The Arabidopsis actin 8 promoter and intron (Genbank accession number U42007 as described in An et al., Plant J. 10:107-121, 1996) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:3 (forward primer) and SEQ ID NO:4 (reverse primer). The promoter was cloned using restriction enzymes as described in Example 2, sequence verified, and the resulting construct was designated pMON26171 (Table 1).

Example 4

[0161] The Arabidopsis actin 11 promoter and intron (Genbank accession number U27981 as described in Huang et al., Plant Mol. Biol., 33:125-139, 1997) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:5 (forward primer) and SEQ ID NO:6 (reverse primer). The promoter was cloned using restriction enzymes EcoRV and NcoI and ligated into pMON8677 (Table 1), sequence verified, and the resulting construct was designated pMON48407 (Table 1).

Example 5

[0162] The Arabidopsis elongation factor 1α (AtEF1α) promoter and intron (Genbank accession number X16430 as described in Axelos et al., *Mol. Gen. Genet.* 219:106-112, 1989; Curie et al., NAR 19:1305-1310; Curie et al., *Plant Mol. Biol.* 18:1083-1089, 1992; Curie et al., *Mol Gen. Genet.* 238:428-436, 1993) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:7 (forward primer) and SEQ ID NO:8 (reverse primer). The promoter was cloned using restriction enzymes HindIII and NcoI and ligated into pMON26152 (Table 1) as described in Example 2, sequence verified, and the resulting construct was designated pMON26177 (Table 1).

Example 6

[0163] The plant transformation constructs described were mated into Agrobacterium. Cotton transformation was performed essentially as described in WO/0036911, herein incorporated by reference in its entirety. The Arabidopsis transformation was performed as described in Ye et al., Plant Journal 19:249-257, 1999. The tomato transformation was performed as described in U.S. Pat. No. 5,565,347 herein incorporated by reference in its entirety.

Example 7

[0164] A DNA construct is transformed into a target crop of interest via an appropriate delivery system such as an Agrobacterium-mediated transformation method (see for example U.S. Pat. No. 5,569,834 herein incorporated by reference in its entirety, U.S. Pat. No. 5,416,011 herein incorporated by reference in its entirety, U.S. Pat. No. 5,631,152 herein incorporated by reference in its entirety, U.S. Pat. No. 5,159,135 herein incorporated by reference in its entirety, U.S. Pat. No. 5,004,863 herein incorporated by reference in its entirety, and U.S. Provisional Appln. No. 60/111795 herein incorporated by reference in its entirety. Alternatively, a particle bombardment method may be used (see for example Patent Applns. WO 92/15675. WO 97/48814 and European Patent Appln. 586,355, and U.S. Pat. Nos. 5,120,657, 5,503,998, 5,830,728 and 5,015,580, all of which are herein incorporated by reference in their entirety).

[0165] A large number of transformation and regeneration systems and methods are available and well-known to those of skill in the art. The stably transformed plants and progeny are subsequently analyzed for expression of the gene in tissues of interest by any number of molecular, immunodiagnostic, biochemical, and/or field evaluation methods known to those of skill in the art, including, but not limited to a spray test with a glyphosate formulation at commercially effective concentrations performed in a growth chamber or field environment.

Example 8

[0166] The GUS assays are performed by routine methods known to those of skill in the art (see for example, Jefferson et al., *EMBO J.* 6:3901, 1987). For cotton, R0 plants were tested. The tissue was size selected at various stages in development, samples and pooled for analysis. The cotton floral bud was harvested and the male reproductive tissue samples (anthers and filaments), female reproductive tissue samples (entire stigma, style, and ovary), and corolla (sepals and petals) were taken. For the size selection, three floral buds from each stage were selected that included several sizes including small (less than 0.5 cm), medium (from 0.5-0.7 cm), and large (candle stage or open flower). Leaf samples were collected about 1-2 weeks after the cotton plants were placed in the greenhouse, and the other samples were collected approximately 1-2 months later. The first flowers were not collected (the first five fruiting positions were left intact).

[0167] For Arabidopsis, V1 plants were analyzed and only homozygous and heterozygous segregants were tested Eight to ten events per construct were analyzed (five plants per event). The GUS results for Arabidopsis represent pooled samples of 8- 10 events. The values in the disclosed tables (Table 2 and Table 3) represent the average GUS expression for the designated tissue (pmol/MU/min/mg).

Example 9

[0168] Plants were analyzed for GUS expression in leaf tissue and reproductive tissues including immature floral buds and flowers. The results are shown in Table 2. Constructs tested included pMON48407 (P-AtAct11+intron/GUS/nos), pMON26170 (P-AtAct2+intron/GUS/nos), pMON26171 (P-AtAct8+intron/GUS/nos), pMON11750 (e35S/GUS/nos), pMON26177 (P-EF1α+intron/GUS/nos), and pMON15737 (P-FMV/GUS/nos). The actin and elongation factor promoters conferred high levels of GUS expression in multiple tissues including reproductive tissues.

TABLE 2

Average Arabidopsis V1 GUS Expression

Construct	Leaf	Immature Floral Bud	Flower	Gynoecium	Androecium
pMON48407	6944	7394	8359	ND	ND
pMON26170	45238	74099	54502	73623	217292
pMON26171	29343	35884	37125	76311	207100
pMON11750	60844	14032	16263	35882	115049
pMON26177	47598	72871	96420	191066	507370
pMON15737	28314	57903	84457	44696	87876

Example 10

[0169] The R0 cotton plants were tested for expression of the GUS reporter gene in selected tissues of various stages of development. The floral buds were staged by size (small, medium, and large; large=candle and open flower). The androecium represented the male reproductive tissues including the entire receptacle (stigma, style, and ovaries). The corolla sample was composed of sepals and petals. The tissue was prepared and GUS assays performed as described in EXAMPLE 8. The results are summarized in Table 3. The constructs tested included pMON48407 (P-EF1α+intron/gus/nos), pMON26170 (P-AtAct2+intron/gus/nos), and pMON48407 (P-AtAct11+intron/gus/nos).

[0170] Six plants were tested and average GUS values obtained for pMON26177. Twenty plants were tested and average GUS values obtained for for pMON26170. Eight plants were tested and average GUS values obtained for pMON48407. The results demonstrate that the actin and elongation factor promoters can be used for effective expression of operably linked genes, particularly in reproductive tissues

TABLE 3

GUS Assay Results for Cotton Plants

Construct	Promoter/intron	Tissue Tested	GUS Results
pMON26177	EF1α	Leaf	11600
pMON26177	EF1α	Small Corolla	396
pMON26177	EF1α	Small Gynoecium	8670
pMON26177	EF1α	Small Androecium	13771
pMON26177	EF1α	Medium Corolla	362
pMON26177	EF1α	Medium Gynoecium	3318
pMON26177	EF1α	Medium Androecium	8006
pMON26177	EF1α	Large Corolla	351
pMON26177	EF1α	Large Gynoecium	500

TABLE 3-continued

GUS Assay Results for Cotton Plants

Construct	Promoter/intron	Tissue Tested	GUS Results
pMON26177	EF1α	Large Androecium	15512
pMON26170	Act2	Leaf	12718
pMON26170	Act2	Small Corolla	1296
pMON26170	Act2	Small Gynoecium	16684
pMON26170	Act2	Small Androecium	7570
pMON26170	Act2	Medium Corolla	742
pMON26170	Act2	Medium Gynoecium	10041
pMON26170	Act2	Medium Androecium	7893
pMON26170	Act2	Large Corolla	289
pMON26170	Act2	Large Gynoecium	3218
pMON26170	Act2	Large Androecium	42737
pMON48407	Act11	Leaf	28289
pMON48407	Act11	Small Corolla	10
pMON48407	Act11	Small Gynoecium	40755
pMON48407	Act11	Small Androecium	47834
pMON48407	Act11	Medium Corolla	742
pMON48407	Act11	Medium Gynoecium	52495
pMON48407	Act11	Medium Androecium	35573
pMON48407	Act11	Large Corolla	1072
pMON48407	Act11	Large Gynoecium	4869
pMON48407	Act11	Large Androecium	42737

Example 11

[0171] Transformed plants were also tested in a greenhouse spray test using Roundup Ultra™ a glyphosate formulation with a Track Sprayer device (Roundup Ultra is a registered trademark of Monsanto Company). Plants were at the "two" true leaf or greater stage of growth and the leaves were dry before applying the Roundup® spray. The formulation used was Roundup Ultra™ as a 3 lb/gallon a.e. (acid equivalent) formulation. The calibration used was as follows:

[0172] For a 20 gallons/Acre spray volume:

Nozzle speed:	9501 evenflow
Spray pressure:	40 psi
Spray height	18 inches between top of canopy and nozzle tip
Track Speed	1.1 ft/sec., corresponding to a reading of 1950—1.0 volts.
Formulation:	Roundup Ultra ™ (3 lbs. A.e./gallon)

[0173] The spray concentrations will vary, depending on the desired testing ranges. For example, for a desired rate of 8 oz/acre a working solution of 3.1 ml/L is used, and for a desired rate of 64 oz/A a working range of 24.8 ml/L is used.

[0174] The evaluation period will vary, depending on the crop, stage of plant development, and tolerance level desired.

Example 12

[0175] The plant expression constructs used for tomato transformation are listed in Table 4. Tomato plants (T0) containing constructs comprising at least one actin or elongation factor promoter (with intron) operably linked to an aroA:CP4 glyphosate tolerance gene are screened in a greenhouse glyphosate spray test with glyphosate (Roundup Ultra™) formulation for the efficiency of conferring glyphosate tolerance to transgenic tomato plants. Optionally, at least one actin or elongation factor promoter sequence operably linked to an aroA:CP4 gene and an eFMV caulimovirus promoter operably linked to an aroA:CP4 transformed into tomato plants are screened by spray application with glyphosate (Roundup Ultra™). Tomato plants are sprayed with 48 oz./acre then evaluated at two weeks post application for analysis of vegetative tolerance and up to 60 days post-application for analysis of reproductive tolerance. The results are shown in Table 4 and ranked according to efficiency of selecting reproductive tolerant lines. The percent vegetative tolerance is the percentage of the lines screened that demonstrated sufficient vegetative tolerance to glyphosate damage to be considered for further studies of agronomic traits in preparation for commercially candidacy. The percent reproductive tolerance is the percentage of the vegetative tolerant lines that also demonstrated sufficient reproductive tolerance to be considered for further agronomic evaluation. All of the constructs proved functional for providing vegetative tolerance and reproductive tolerance to the transgenic tomato plants. Various combinations of promoters are able to increase the efficiency at which vegetative and reproductive tolerant lines could be selected by screening in this experiment. Constructs containing the Arabidopsis EF1α promoter are more specifically associated with a high percentage of vegetatively tolerant lines. P-Act2 promoter in combination with P-eFMV and P-AtEF1α (pCGN9190) provided an increase in the percentage of reproductively tolerant lines that are screened by this method.

TABLE 4

Greenhouse Track Spray Trials with Application Rate of 48 oz./Acre*

Construct	Description	# Lines Tested	% Vegetative Tolerance[1]	% Reprod. Toler.[2]
pCGN9190	eFMV/CP4 + EF1α/CP4 + Act2/CP4	930	83.2	52.4
pCGN9153	EF1α/CP4 + eFMV/CP4	391	73.9	38.9
pCGN8086	Act8/CP4	21	47.6	38.1
pCGN8099	EF1α/CP4 + Act8/CP4	71	84.5	36.6
pCGN8088	eFMV/CP4 + Act8/CP4	144	79.9	34.7
pMON45325	eFMV/CP4 + Act11/CP4	90	70.0	34.4
pCGN8096	Act11/CP4 + Act2/CP4	201	62.7	10.4
pCGN8067	Act2/CP4	205	67.3	8.8

*one application

[1]Pooled Results from 25 screens. Scored 14 days post-application

[2]Pooled Results from 25 screens. Scored up to 60 days post-application

[0176] Tomato seed yield is used as a measure of the efficacy of the various promoter sequences and combination of expression cassettes used in the present invention for conferring glyphosate tolerance to transgenic tomato plants. In Table 5, the results of three field experiments are shown on transgenic tomato plants containing constructs with the promoters of the present invention driving expression of the aroA:CP4 coding sequence for glyphosate tolerance. Experiment I is a test of the plants produced from the constructs that contain the Figwort mosaic virus promoter (P-FMV) in the native and the duplicated enhanced version (P-eFMV) and additional genetic elements in the constructs that are

also found in the constructs used to test the promoter sequences of the present invention. Additional genetic elements such as the source of the 5' untranslated sequence and the chloroplast transit peptide are also tested. The construct pMON20998 comprises the P-eFMV, linked to the petunia Hsp70 5' UTR, leader linked to the Arabidopsis EPSPS chloroplast transit peptide (CTP2), linked to the E9 3' termination region. The construct pMON20999 differs from pMON20998 only in that the promoter is P-FMV. The construct pMON10156 differs from pMON20998 only in that the CTP is from the Petunia EPSPS chloroplast transit peptide (CTP4). The construct pMON45312 differs from pMON20998 only in that the leader sequence is the native FMV leader sequence.

[0177] Tomato plants are transplanted into the field in rows. The plants are spray treated in the field at a rate of 48 oz./Acre with Roundup herbicide. The tomato seed is collected from the fruit and weighted. An unsprayed tomato line serves as the control for comparison purposes and the efficacy of each construct is expressed as a percentage of the control. The result of Experiment 1 (column 1 of Table 5) is that the FMV promoter and P-eFMV only provide 5-11 % of the seed production of an unsprayed check. Experiment 2, and 3 tests the constructs of the present invention at different locations (columns 2 and 3 of Table 5). Experiment 2 is conducted at the same location as Experiment 1, the constructs pCGN8099 (**FIG. 7**), pCGN9151 (**FIG. 11**) and pCGN9190 (**FIG. 5**) performed well by providing 25-46% of the seed relative to an unsprayed check. At a different location that has a cooler growing season, Experiment 3 demonstrated that pCGN8068 (**FIG. 9**), pCGN8088 (**FIG. 8**), pCGN8099, pCGN9151, pCGN9153 (**FIG. 6**), and pMON45325 (**FIG. 2**) are able to confer sufficient glyphosate tolerance for the tomatoes to set 34-77% of normal seed set relative to an unsprayed check.

TABLE 5

Tomato seed yield experiments

	Exp. 1		Exp. 2		Exp. 3	
	Seed wt grams	% of Control	Seed wt grams	% of Control	Seed wt grams	% of Control
pMON20998	0.52	5.3				
pMON20999	0.84	8.6				
pMON10156	0.50	5.1				
pMON45312	1.07	11.0				
pCGN8068			0.48	8.4	7.06	77.8
pCGN8088			0.43	7.6	3.09	34.1
pCGN8096			0.40	7.0		
pCGN8099			1.85	32.5	6.93	76.4
pCGN9151			1.46	25.7	6.11	67.4
pCGN9153			0.68	12.0	4.03	44.4
pCGN9190			2.64	46.4		
pMON45325			0.31	5.4	3.37	37.2
pCGN8067						
Control	9.73	100.0	5.69	100.0	9.07	100.0

Example 13

[0178] SEQ ID NOS: 1-8, and SEQ ID NOS: 13-21 are PCR primers designed from publicly available sequence information for *Arabidopsis thaliana* Act 1, Act2 (Genbank #U41998), Act3, Act7, Act8 (Genbank #ATU42007), Act11 (Genbank #ATU2798 1), Act12 and Elf1α (Genbank #X16430) genes. These sequences are used to extend the nucleic acid sequence using polymerase chain reaction (PCR) amplification techniques (see for example, Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51:263, 1986; Erlich, et al., European Patent Appln. 50,424; European Patent Appln. 84,796, European Patent Appln. 258,017, European Patent Appln. 237,362; Mullis, European Patent Appln. 201,184; Mullis, et al., U.S. Pat. No. 4,683,202; Erlich, U.S. Pat. No. 4,582,788; and Saiki, et al., U.S. Pat. No. 4,683,194). A number of PCR amplification methods are known to those of skill in the art and are used to identify nucleic acid sequences adjacent to a known sequence. For example, inverse PCR (IPCR) methods, which are used to amplify unknown DNA sequences adjacent to a core region of known sequence have been described. Other methods are also available such as capture PCR (Lagerstrom M., et al., PCR Methods Applic. 1:111, 1991), and walking PCR (Parker, et al., Nucleic Acids Res 19:3055, 1991). A number of manufacturers have also developed kits based on modifications of these methods for the purposes of identifying sequences of interest. Technical advances including improvements in primer and adaptor design, improvements in the polymerase enzyme, and thermocycler capabilities have facilitated quicker, efficient methods for isolating sequences of interest.

TABLE 5

Primer sequences for isolation of *Arabidopsis Actin* and EF1α promoter sequences

At. Actin 2 forward:
TTTTTTTTGATATCAAGCTTCAACTATFITJ7ATGTATGC
At. Actin 2 reverse:
GCCTCAGCCATGGTGAGTCTGCTGCAAACACACAAAAAGAGTTCAAT
At. Actin 8 forward:
TTTTTTTTGATATCAAGCTTCCATTTTTCT TTTGCATAAT TC
At. Actin 5 reverse:
GCATCGGCCATGGTGAGTCTTCTGCAATCAAAAACATAAAGATCTGA
At. Actin 11 forward:
TTTTTTTTTAAGCTTGATATCACAACCAAATGTCAAATGG
At. Actin 11 reverse:
CCATCTGCCATGGTCTATATCCTGTC
At. EF1α forward:
TTTTTTTTTAAGCTTGATATCGGAAGTTTCTCTCTTG
At. EF1α reverse:
CTTTTCCCATGGTAGATCTCTGGTCAACAA ATC
At. Actin 1a forward:
CCCAAGCTTAAATGACATCAGATACACGC
At. Actin 1b forward:
CATAAGCTTAGAGGTCCAAATTCA
At. Actin 1 reverse:
CCATCAGCCATGGTCTTCTACCTTTATGCAAA
At. Actin 3 forward:
CCAAGCTTACCACACTCAGATGCATAAACAAACACA
At. Actin 3 reverse:
CATCAGCCATGGTCTACTCTCTGCAAAAACA
At. Actin 7 forward:
GCAAAGCTTACTAGTCAACAATTGGCC

TABLE 5-continued

Primer sequences for isolation of *Arabidopsis Actin* and EF1α promoter sequences

At. Actin 7 reverse:
GATCGGCCATGGTTCACTAAAAAAAAAG

At. Actin 12 forward:
GGAAGCTTGCGGCCGCTTTCTACTCTACATGTTTCT

At. Actin 12 reverse:
GACTAGCCGCCATGGTTCAATCTCTAGCTGA

[0179] The leaves of young plants of *Arabidopsis thaliana* (1 g) were homogenized in 9 ml of CTAB buffer (Saghai-Maroof et al. 1984, PNAS 81:8014-8018). The CTAB buffer contained 100 mM TrisHCl; pH 7.8, 700 mM NaCl, 50 mM EDTA, 1% CTAB (alkytrimethyhylammoniumbromide) and 140 mM 2-mercaptoethanol. After 90 minutes incubation in 65 C, 4.5 ml of chloroform:isoamyl alcohol (24: 1) was added and samples were mixed for 10 minutes. Aqueous layer was separated by centrifugation for 10 minutes at 1500 g and was re-extracted with chloroform:isoamyl alcohol. After second centrifugation, aqueous layer was transferred to a tube containing 50 μl 10mg/ml RNase A (DNase free) and incubated in room temperature for 30 minutes to remove RNA. DNA was precipitated with 6 ml of isopropanol and re-suspended in 1 ml of 10 mM TrisHCl buffer pH 8.5. DNA solution was extracted once with equal volume of phenol and once with an equal volume of chloroform: isoamylalcohol. After centrifugation, 1/10 volume of sodium acetate (3M, pH 5.2) was added to aqueous layer, followed by 2.5 volume of ethanol. The DNA was hooked, washed in 70% ethanol, then air dried and re-suspended in 0.2 ml of 10 mM TrisHCl buffer.

[0180] Arabidopsis genomic DNA (100 ng) was used in 50 μl PCR reactions. Reactions containing the primers shown in Table 5. contained 10 μM reverse and forward primer solutions, 200 nM dNTPs and PCR buffer with magnesium and DNA polymerase mix from Expand™ High Fidelity PCR System (Roche Molecular Biochemicals). After initial 2 minute denaturation at 94° C. reactions were cycled 0.5 min at 94° C., 0.5 min at 55° C. and 1.5 minute at 72° C. for 35 times. PCR products were analyzed by electrophoresis on 1% agarose gel. Gel isolated DNA fragments representing Actin 1a, Actin 1b, Actin 7, and Actin 12 sequences were phosphorylated with T4 DNA kinase and ligated to dephosphorylated and Sma I cut pUC19 cloning construct. White colonies were screened for the presence of appropriate inserts and sequenced with M13 forward and reverse primers to confirm the presence of actin promoters. Selected clones were designated as pMON54941 (P-AtAct1a), pMON54942(P-AtAct1b), pMON54943 (P-AtAct7) and pMON54944 (P-AtAct12). Subsequently, the Actin promoters DNA fragments were released by Hind III and NcoI digest of the pUC19 constructs containing the insert sequences, the DNA fragments were gel isolated and ligated to pMON26165 that had been digested with the same restriction enzymes. A PCR product for the Actin 3 promoter (P-AtAct3) was digested with Hind III and Nco I and cloned directly into pMON26165 to form pMON5495 1. pMON26165 contains the GUS/nos terminator gene segment. Ligation with the promoter segments allows for assay of each promoter for functional activity by expression of the β-glucuronidase enzyme in plant cells. The plant cells can be isolated, for example, tobacco leaf protoplasts, or the plant cells may be contained in a plant tissue or organ, such as, leaf, root, cotyledon, hypocotyl, embryo, flower, or storage organ.The expression level of GUS driven by these promoters is assayed in soybean hypocotyl in comparison with GUS driven by P-e35S promoter (Table 6). Plasmid DNA/gold particles was bombarded to soybean hypocotyls then after 48 hours the GUS activity was assayed histochemically. All of the Actin promoters tested in this assay show functional activity in the hypocotyl tissue demonstrating their utility for expression transgenes in heterologous crop plant species.

[0181] The constructs containing aroA:CP4 EPSPS driven by the Arabidopsis Actin 1a, (pMON54952), Actin 1b (pMON54953), Actin 3 (pMON54954), Actin 7 (pMON54955) and Actin 12 (pMON54956) promoters of the present invention were prepared in Agrobacterium binary plant transformation constructs for stable expression of the glyphosate resistant EPSPS in crop plants. These constructs are transformed into soybean and cotton cells, the cells are selected and regenerated into plants on glyphosate containing tissue culture media and then assayed for expression of the aroA:CP4 protein and for tolerance to glyphosate application. Plants demonstrating commercially acceptable glyphosate tolerance are further developed by conventional breeding methods to transfer the glyphosate tolerance trait into germplasm adapted for cultivation.

TABLE 6

Activity of different Arabidopsis actin promoters in transient assay as compare to P-e35S.

Construct	GUS Activity
Pe35S/GUS	+++
P-AtAct1a/GUS	++
P-AtAct1b/GUS	++
P-AtAct3/GUS	++
P-AtAct7/GUS	++
P-AtAct12/GUS	+

Example 14

[0182] Cotton yield is correlated with the number of squares set during the first four to five weeks of squaring. The retention of these squares to mature bolls and their contribution to the harvest of the cotton lint is a key component of yield. When determining the efficacy of transgene constructs for conferring herbicide tolerance in cotton, the amount of boll retention is a measure of efficacy and is a desirable trait. Transgenic cotton plants containing promoters of the present invention (Table 7) were assayed in greenhouse conditions for boll retention. The promoters directed expression of the aroA:CP4 coding sequence for glyphosate tolerant phenotype. The plants were transformed by an Agrobacterium-mediated method or by a particle gun method. The particle gun constructs contained an additional GUS containing expression cassette useful for histochemical localization of β-glucuronidase activity from the promoters of the present invention. Transgenic plants were regenerated on glyphosate containing media and plants rooted on a rooting media. The rooted plantlets were potted in soil and transferred to a growth chamber for a hardening off period.

The seed from these plant lines were collected and planted. Fifteen plants from each line were sprayed with glyphosate at 48 ounces/acre at the 4 leaf stage. At least 8 surviving plants from each line were sprayed again at the 8 leaf stage with glyphosate at 48 ounces/acre. At maturity, the number of first position bolls for the first five bolls was counted. Those lines that had 3 or more of the first position bolls retained after the glyphosate spray (plant map≧3) were advanced for further study. Table 7 illustrates the data produced from this study. The number of lines mapped indicates the number of lines surviving the first glyphosate spray application. The commercial standard is Line 1445 (pMON17136) that contains the P-FMV promoter driving expression of the CTP2-aroA:CP4 gene/E9 3', this line retains less than 1 of the 5 first bolls. The constructs, pCGN8099, pCGN9153, pCGN8088, pCGN8068 provided sufficient reproductive glyphosate tolerance in cotton such that 14-35% of the lines tested from these constructs were advanced for further agronomic trials.

TABLE 7

Greenhouse cotton boll retention study

Construct	Promoters	# lines Mapped	Plant Map ≧ 3	% ≧ 3
pCGN8099	eFMV:EF1a + e35S:Act8	104	36	34.6%
pCGN9153	EF1α + FMV	36	12	33.3%
pCGN9165	EF1α + 35S/GUS	3	1	33.3%
pCGN9152	EF1a	7	0	0.0%
pCGN8088	Act8 + FMV	43	6	14.0%
pCGN8086	Act8	7	0	0.0%
pCGN8068	Act2 + FMV	37	7	18.9%
pCGN8067	Act2	37	0	0.0%
pCGN8084	Act2 + FMV + 35S/GUS	5	0	0.0%
pCGN8085	Act2 + FMV/GUS	1	0	0.0%
pCGN9164	Act11 + 35S/GUS	21	1	4.8%
pMON45325	Act11 + FMV	43	0	0.0%
pCGN8096	eFMV:Act11 + e35S:Act2	14	0	0.0%
pCGN9154	eFMV:Act11 + e35S:Act2	16	1	6.3%
Line 1445	FMV		<1.0	

Example 15

[0183] Cotton yield is correlated with the number of squares set during the first four to five weeks of squaring. The retention of these squares to mature bolls and their contribution to the harvest of the cotton lint is a key component of yield. When determining the efficacy of transgene constructs for conferring herbicide tolerance in cotton, the amount of boll retention is a measure of efficacy and is a desirable trait. Transgenic cotton plants containing promoters of the present invention were assayed in field conditions at two locations for boll retention. The transgenic cotton lines 502-254-2 (pCGN8068), 701-178-2 (pCGN8068), 53-2 (pCGN8088), 178-1 (pCGN9153), and 60-1 (pCGN9153) were compared to 1445 (glyphosate tolerance line) and PM1218BR (Paymaster 1218 parent) that contain the construct pMON17136 (P-FMV/CTP2-aroA:CP4/E9'), a wild type non-transgenic line, Coker 130 was included. The field design is a randomized complete block design consisting of 2 rows×20-30 feet×3 replications. Glyphosate is applied as Roundup Ultra™ formulation at rates of 1.12 lb ai/A=48 oz product and 1.5 lb ai/A=64 oz product at the 8 leaf stage of cotton plant development. All of the cotton plots are managed aggressively for weed and insect pest control, as well as other agronomic inputs such as planting time, fertilization, irrigation, PGR usage and defoliation. The percent boll retention is determined by mapping the location of each of the retained bolls by random selection of ten plants from the middle of the two center rows (five from each row) of each plot to map. The first mapping should be done 4 weeks after first flower (mid-season map), a second mapping should be done at harvest. The data collected includes the number of first position bolls on the bottom five flowering nodes that are counted as an indication of the reproductive tolerance of the transgenic cotton lines to glyphosate. Table 8 illustrates the advantage that promoters of the present invention have conferred to transgenic cotton plants for boll retention. This enhanced reproductive tolerance has resulted in increased lint yield (Table 9) and increased seed yield (Table 10) as well.

TABLE 8

Boll retention at mid-season plant map of bottom 5 first position boils

	Location 1			Location 2		
	Untreated	48 oz/A	64 oz/A	Untreated	48 oz/A	64 oz/A
(17136) 1445	68	67	53	81	63	62
(8068) 502-254-2	87	72	64	77	80	69
(8068) 701-178-2	85	77	60	84	86	76
(8088) 53-2	89	81	80	79	76	73
(9153) 178-1	77	83	73	85	71	79
(9153) 60-1	80	89	81	77	82	87
PM1218BR	92	56	63			

[0184]

TABLE 9

Lint Yield (lbs/Acre) and percent yield (Location 1)

Cultivar	Untreated	48 oz/A	48 oz/A %	64 oz/A	64 oz/A %
8068-502-254-2-4	1103	960	87.0%	858	77.8%
8068-701-178-2-2	1326	1219	91.9%	1177	88.8%
9153-60-1-1	1177	1206	102.5%	1171	99.5%
9153-178-1-1	1112	769	69.2%	750	67.4%
8088-53-2-11	1283	1071	83.5%	1097	85.5%
1445	1018	563	55.3%	490	48.1%
C130	1200	0	0.0%	0	0.0%
PM 1218 BR	1092	826	75.6%	713	65.3%

[0185]

TABLE 10

Seed Cotton Yield (lbs/Acre) and percent yield (Location 1)

Cultivar	Untreated	48 oz/A	48 oz/A %	64 oz/A	64 oz/A %
8068-502-254-2-4	3357	2923	87.1%	2646	78.8%
8068-701-178-2-2	3720	3521	94.7%	3328	89.5%
9153-60-1-1	3294	3413	103.6%	3316	100.7%
9153-178-1-1	3468	2355	67.9%	2218	64.0%
8088-53-2-11	3404	2950	86.7%	2968	87.2%
1445	2835	1624	57.3%	1372	48.4%
C130	3272	0	0.0%	0	0.0%
PM 1218 B/RR	3036	2192	72.2%	1885	62.1%

Example 16

[0186] The efficacy of the hybrid promoter P-eFMV-AtEF a driving expression of the CTP2-aroA:CP4 coding sequence (**FIG. 13**, pMON52059) and P-FMV/CTP2-aroA:CP4/E93' (pMON15737) was compared in transgenic *Arabidopsis thaliana*. The transgenic *Arabidopsis thaliana* plants were produced by the vacuum infiltration (Bechtold et al., C R Acad Paris Life Sci 316: 1194-1199) seeds were potted in soil in trays in a growth chamber adjusted for 24° C., 16 hour light (120 μE $m^{-2}s^{-1}$) cycle to permit normal growth and development of the plants. The pMON52059 V1 event glyphosate tolerant transgenic Arabidopsis plants were selected by spray application of glyphosate herbicide at a rate of 24 ounces/acre, the surviving plants were transplanted into individual pots. Eight pMON52059 V1 plants and eight pMON15737 homozygous plants were sprayed a second time corresponding to the observation of bolting, approximately 16 days after the at a rate of 24 ounces/acre. The second spray will determine the efficacy of the two constructs for conferring reproductive tolerance. The plants were observed for vegetative effects of glyphosate application. All plants had complete vegetative tolerance and no abnormal flowers were observed. However, abortion of siliques occurred indicated that seed had not been set in the aborted siliques. The total number of siliques produced by each plant and the siliques that contained seeds (fertile siliques) were counted and tabulated. The results are shown in Table 9 and indicate that the hybrid promoter construct pMON52059 demonstrated a greater than 10 fold improvement in fertile siliques, 89% compared to pMON15737 at 8%. The number of fertile fruiting structures is related to the amount of seed that can be produced, this is especially important in crops whose yield is associated with seed numbers. Crops such as cotton, soybean, canola, wheat, and corn are crops where reproductive tolerance to glyphosate is essential for good yield.

Example 19

[0187] Sunflower (*Helianthus annuus L.*) is a crop of agronomic importance for oil and food. The constructs pMON45325 (**FIG. 2**), pMON45332 (**FIG. 4**), and pMON45331 (**FIG. 3**) of the present invention were transformed into sunflower. Agrobacterium-mediated transformation of sunflower has been reported (Schrammeijer et al., *Plant Cell Reports*, 9: 55-60, 1990; EP 0 486 234. Methods known by those skilled in the art of plant transformation with transgene expression constructs can include hypocotyls, apical meristems, protoplasm, and other sunflower tissues. Transgenic sunflower lines SFB250-27 contains pMON20999 (P-FMV/CTP2-aroA:CP4/E93') expression cassette; SFB288-01, SFB295-09 contain pMON45325 (P-eFMV/CTP2-aroA:CP4/E93':P-AtAct11+intron/CTP2-aroA:CP4/E93'); SFB289-01 contains pMON45332 (P-AtEF1α+intron/CTP2-aroA:CP4/E93':P-eFMV/CTP2-aroA:CP4/E93'); SFB303-08, SFB303-09, SFB303-11, and HA300B contain pMON45331 (P-AtEF1α+intron/CTP2-aroA:CP4/E9). These lines are tested for glyphosate tolerance and are shown in Table 12.

[0188] The reproductive tolerance to glyphosate in sunflower can be measured as a function of the percent of normal heads, percent normal head size and the pollen production. These plants are sprayed with Glyphosate at V-4 and V-8 leaf stages at 0, 32 oz/acre or 64 ounces/acre rate. The sunflower plants are assessed for vegetative tolerance to glyphosate.. Vegetative tolerance is achieved at 32 and 64 oz/acre levels of glyphosate spray at both V4 and V8 stages of plant development.

[0189] Vegetative glyphosate tolerant transgenic sunflower lines are scored for number of heads, percent normal heads, percent normal head size, and percent normal pollen shed. These traits are scored in a field test at one location. The tabulation of the head scores and pollen production is

TABLE 11

Comparison of the hybrid promoter P-eFMV-EF1α (pMON52059) and P-FMV (pMON15737) in conferring reproductive tolerance to glyphosate in *Arabidopsis* plants.

pMON52059				pMON15737			
Plant Number	Fertile Siligues	Total Siligues	Percent Fertility	Plant Number	Fertile Siligues	Total Siligues	Percent Fertility
8819	39	50	78.0%	1	74	540	13.7%
8820	626	691	90.6%	2	23	600	3.8%
8821	507	561	90.4%	3	1	470	0.2%
8822	0	69	0.0%	4	20	646	3.1%
8823	512	534	95.9%	5	43	717	6.0%
8827	326	354	92.1%	6	22	651	3.4%
8833	432	461	93.7%	7	178	86820.5%	
8838	323	374	86.4%	8	40	520	7.7%
Total	2765	3094	89.4%	Total	401	5012	8.0%

BNSDOCID: <US____20030199681A1_I_>

shown in Table 12. Lines selected from the constructs of the present invention show greater percent of normal heads, generally greater percent normal head size and better pollen shed.

TABLE 12

Sunflower glyphosate resistance scores

Line #	# heads	% normal heads	% normal head size	% pollen shed
SFB250-27	28	29	75	36
SFB288-01	11	36	73	73
SFB295-09	28	57	64	68
SFB289-01	13	38	92	38
SFB303-08	25	68	92	64
SFB303-09	43	81	88	88
SFB305-11	45	71	84	100
HA300B	30	100	97	97
non-trans segregant	0	0	0	0

Example 18

[0190] Cis acting regulatory elements necessary for proper promoter regulation can be identified by a number of means. In one method, deletion analysis is carried out to remove regions of the promoter and the resulting promoter fragments are assayed for promoter activity. DNA fragments are considered necessary for promoter regulation if the activity of the truncated promoter is altered compared to the original promoter fragment. Through this deletion analysis, small regions of DNA can be identified which are necessary for positive or negative regulation of transcription. Promoter sequence motifs can also be identified and novel promoters engineered to contain these cis elements for modulating expression of operably linked transcribable sequences. See for example U.S. Pat. No. 5,223,419, herein incorporated by reference in its entirety, U.S. Pat. No. 4,990,607 herein incorporated by reference in its entirety, and U.S. Pat. No. 5,097,025 herein incorporated by reference in its entirety.

[0191] An alternative approach is to look for similar sequences between promoters with similar expression profiles. Promoters with overlapping patterns of activity can have common regulatory mechanisms. Several computer programs can be used to identify conserved, sequence motifs between promoters, including but not limited to MEME, SIGNAL SCAN, or GENE SCAN. These motifs can represent binding sites for transcriptions factors which act to regulate the promoters. Once the sequence motifs are identified, their function can be assayed. For example, the motif sequences can be deleted from the promoter to determine if the motif is necessary for proper promoter function. Alternatively, the motif can be added to a minimal promoter to test whether it is sufficient to activate transcription. Suspected negative regulatory elements can be tested for sufficiency by adding to an active promoter and testing for a reduction in promoter activity. Some cis acting regulatory elements may require other elements to function. Therefore, multiple elements can be tested in various combinations by any number of methods known to those of skill in the art.

[0192] Once functional promoter elements have been identified, promoter elements can be modified at the nucleotide level to affect protein binding. The modifications can cause either higher or lower affinity binding which would affect the level of transcription from that promoter.

[0193] Promoter elements can act additively or synergistically to affect promoter activity. In this regard, promoter elements from different 5' regulatory regions can be placed in tandem to obtain a promoter with a different spectrum of activity or different expression profile. Accordingly, combinations of promoter elements from heterologous sources or duplication of similar elements or the same element can confer a higher level of expression of operably linked transcribable sequences. For example, a promoter element can be multimerized to increase levels of expression specifically in the pattern affected by that promoter element.

[0194] The technical methods needed for constructing expression constructs containing the novel engineered 5' regulatory elements are known to those of skill in the art. The engineered promoters are tested in expression constructs and tested transiently by operably linking the novel promoters to a suitable reporter gene such as GUS and testing in a transient plant assay. The novel promoters are operably linked to one or more genes of interest and incorporated into a plant transformation construct along with one or more additional regulatory elements and transformed into a target plant of interest by a suitable DNA delivery system. The stably transformed plants and subsequent progeny are evaluated by any number of molecular, immunodiagnostic, biochemical, phenotypic, or field methods suitable for assessing the desired characteristic(s).

[0195] Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

[0196] All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 30

<210> SEQ ID NO 1
<211> LENGTH: 39
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<213> ORGANISM: artificial sequence

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<222> LOCATION: (1)..(39)
<223> OTHER INFORMATION: fully synthetic sequence

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<213> ORGANISM: artificial sequence
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<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(47)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 2

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<210> SEQ ID NO 3
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<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(42)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 3

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<210> SEQ ID NO 4
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(47)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 4

gcatcggcca tggtgagtct tctgcaatca aaaacataaa gatctga 47

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<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(40)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 5

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<210> SEQ ID NO 6
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(26)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
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<212> TYPE: DNA
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<222> LOCATION: (1)..(37)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 7

ttttttttta agcttgatat cggaagtttc tctcttg 37

<210> SEQ ID NO 8
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(33)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 8

cttttcccat ggtagatctc tggtcaacaa atc 33

<210> SEQ ID NO 9
<211> LENGTH: 1219
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1219)
<223> OTHER INFORMATION: Act2 promoter polynucleotide sequence and intron

<400> SEQUENCE: 9

caactatttt tatgtatgca agagtcagca tatgtataat tgattcagaa tcgttttgac 60

gagttcggat gtagtagtag ccattattta atgtacatac taatcgtgaa tagtgatatg 120

atgaaacatt gtatcttatt gtataaatat ccataaacac atcatgaaag acactttctt 180

tcacggtctg aattaattat gatacaattc taatagaaaa cgaattaaat tacgttgaat 240

tgtatgaaat ctaattgaac aagccaacca cgacgacgac taacgttgcc tggattgact 300

cggtttaagt taaccactaa aaaaacggag ctgtcatgta acacgcggat cgagcaggtc 360

acagtcatga agccatcaaa gcaaaagaac taatccaagg gctgagatga ttaattagtt 420

taaaaattag ttaacacgag ggaaaaggct gtctgacagc caggtcacgt tatctttacc 480

tgtggtcgaa atgattcgtg tctgtcgatt ttaattattt ttttgaaagg ccgaaaataa 540

agttgtaaga gataaacccg cctatataaa ttcatatatt ttcctctccg ctttgaattg 600

tctcgttgtc ctcctcactt tcatcagccg ttttgaatct ccggcgactt gacagagaag 660

aacaaggaag aagactaaga gagaaagtaa gagataatcc aggagattca ttctccgttt 720

tgaatcttcc tcaatctcat cttcttccgc tctttctttc caaggtaata ggaactttct 780

ggatctactt tatttgctgg atctcgatct tgttttctca atttccttga gatctggaat 840

tcgtttaatt tggatctgtg aacctccact aaatcttttg gttttactag aatcgatcta 900

agttgaccga tcagttagct cgattatagc taccagaatt tggcttgacc ttgatggaga 960

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gatccatgtt catgttacct gggaaatgat ttgtatatgt gaattgaaat ctgaactgtt 1020

gaagttagat tgaatctgaa cactgtcaat gttagattga atctgaacac tgtttaagtt 1080

agatgaagtt tgtgtataga ttcttcgaaa ctttaggatt tgtagtgtcg tacgttgaac 1140

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ttgcagcaga ctcaccatg 1219

<210> SEQ ID NO 10
<211> LENGTH: 1271
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1271)
<223> OTHER INFORMATION: y = t/u or c
Act8 promoter polynucleotide sequence and intron

<400> SEQUENCE: 10

ccatttttct tttgcataat tcatgtttat ttttttattt ttttcatctt gcataattca 60

tgtttaaaag gatatataca tgggtctact acattctcct gacattacgt tttatgtgtt 120

tgtcttctga aaataatcat caaaatattt caggacttgt ttacgttttc aggagaaaaa 180

aaataactgt acccttttca atatagaaat aacatttgta gaaatcgtgg attttcctta 240

ataaacaatc caaaacacga ccaccgttgt ctcctcgact cggtaacacc cgatcgccga 300

cttgaaaatt agaagaaaaa tgaaaagaat aataaaaaaa aaaaaggaat gatgattgaa 360

gctgtcatat atgtcgaccc tatcacagtc aatccaatag cctatattcg ccaactgata 420

tatccaacgg ctcacaaatt ttcacaaact tttcaaaaaa gtataataaa agaggctgtc 480

tgacagccat gtcacgttat acttttccg tatgatcgaa atgattcgtc tttgyygaat 540

ttaattattt ccaaaattga ygactctaaa gaaaaaaaaa tagtttttca gataaacccg 600

cctatataaa tagttcaaca ctcggtttat ttcttctccc ctcaaagaat tgcctcgtcg 660

tcttcagctt catcggccgt tgcatttccc ggcgataaga gagagaaaga ggagaaagag 720

tgagccagtt cttcatcgtc gtggttcttg tttcttcctc gatctctcga tcttctgctt 780

ttgcttttcc gattaaggta attaaaacct ccgatctact tgttcttgtg ttggatctcg 840

attacgattt ctaagttacc ttcaaaagtt gtttccgatt tgattttgat tggaatttag 900

atcggtcaaa ctattggaaa tttttgatcc tggcaccgat tagctcaacg attcatgttt 960

gacttgatct tgcgttgtat ttgaaatcga tccggatcct ttcgcttctt ctgtcaatag 1020

gaatctgaaa tttgaaatgt tagttgaagt ttgacttcag attctgttga tttattgact 1080

gtaacatttt gtcttccgat gagtatggat tcgttgaaat ctgctttcat tatgattcta 1140

ttgatagata catcatacat tgaattgaat ctactcatga atgaaaagcc tggtttgatt 1200

aagaaagtgt tttcggtttt ctcgatcaag attcagatct ttatgttttt gattgcagat 1260

cgtagaccat g 1271

<210> SEQ ID NO 11
<211> LENGTH: 1393
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1393)
<223> OTHER INFORMATION: Act11 promoter polynucleotide segeunce and

-continued

intron

<400> SEQUENCE: 11

acaaccaaat gtcaaatgga atgcatcaga gaccaaacct gtaagagtcc acaaaacaat 60

tcaaagaaag aatatcaaca attcagagat tcaatcctaa aacaaaaaga gaactgaaac 120

caaatcgtac ctacacgacc agtgaagata ccaatagaga gctctgttgt agaatacaac 180

acattaagcg caattagcag aaacagtctc ttcatctgcc gatttccact tgtcactact 240

ccaaaaacct cccaaaccat ttccaaaaca gacacttttg ccatgtctac atctttccct 300

tccccgaaaa acacatcatt tccatcaacg gagtaaatat ccggcggcat atcgatgctc 360

gagaccgtcc tatcgagaaa aggcttagcc gcttccgtga ccgccggcgt tcgtggaccg 420

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tcgctgctcc gatcatcagg gagctcgtta tcgcctcgag gattaaagga aatggatctc 540

tccatttttct tctttgatct taaagttcca acttcggcaa atactaaaat caacagtcag 600

tcgtacaaag aaactctgct tatacagtaa agtcaatggg ccactgttct aagcccatat 660

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aacaattgtt aaaaagggtt ggttagtgtg tatgtatata tgaaatgcaa caaacattat 780

acagcccatt aaatatggtt gttataggta gatgtcccca ttaaggaact ttatccagcc 840

cattaaatta ctttacagag taaaagagag agagaagatt tacagttacg ttaccaaatt 900

ttcgaaatga tttaattagt aataaataaa taattaaatg tcagttactc tctttagaaa 960

gctaaataag acagctgttt ccaccaacaa cgtgactggt cgtggggtcc tccttcgttc 1020

aaagtgatat tcagaaatca acggctgaga tcttctccat caatatttat tacgggccta 1080

ttccttcctt ttttaaactt caattctccg gctcacattc tcttcttcat tcgctccgtt 1140

tctctctcaa aaactacaca cccgtaccac accaccaccc tcctcgtttc ctcagagatc 1200

ccctctctaa cttctaaggt aatcacattt ccataacgtt ccatcgtcat tgattcttca 1260

ttagtatgcg tttatgaagc tttttcaatt taattctctt tggtagatct taagattcct 1320

ctgtttcttg caaaataaag ggttcaatta tgctaatatt ttttatatca attttgacag 1380

gatatagacc atg 1393

<210> SEQ ID NO 12
<211> LENGTH: 1160
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1160)
<223> OTHER INFORMATION: r = g or a, y = t/u or c, n = a or g or c or t/u EF1 promoter polynucleotide sequence and intron

<400> SEQUENCE: 12

ggaagtttct ctcttgaggg aggttgctcg tggaatggga cacatatggt tgttataata 60

aaccatttcc attgtcatga gattttgagg ttaatatata ctttacttgt tcattatttt 120

atttggtgtt tgaataaatg atataaatgg ctcttgataa tctgcattca ttgagatatc 180

aaatatttac tctagagaag agtgtcatat agattgatgg tccacaatca atgaaatttt 240

tgggagacga acatgtataa ccatttgctt gaataacctt aattaaaagg tgtgattaaa 300

tgatgtttgt aacatgtagt actaaacatt cataaaacac aaccaaccca agaggtattg 360

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agtattcacg gctaaacagg ggcataatgg taatttaaag aatgatatta ttttatgtta 420

aaccctaaca ttggtttcgg attcaacgct ataaataaaa ccactctcgt tgctgattcc 480

atttatcgtt cttattgacc ctagccgcta cacacttttc tgcgatatct ctgaggtaag 540

cgttaacgta cccttaratc gttcyttttc yttttcgtct gctgatcgtt gctcatatta 600

tttcgatgat tgttggattc gatgctcttt gttgattnat cgttctgaaa attctnatct 660

gttgtttaga ttttatcgat tgttaatatc aacgtttcac tgcttctaaa cgataattta 720

ttcatgaaac tattttccca ttctgatcga tcttgttttg agattttaat ttgttcgatt 780

gattgttggt tggtggatct atatacgagt gaacttgttg atttgcgtat ttaagatgta 840

tgtcgatttg aattgtgatt gggtaattct ggagtagcat aacaaatcca gtgttccctt 900

tttctaaggg taattctcgg attgtttgct ttatatctct tgaaattgcc gatttgattg 960

aatttagctc gcttagctca gatgatagag caccacaatt tttgtggtag aaatcggttt 1020

gactccgata gcggcttttt actatgattg ttttgtgtta aagatgattt tcataatggt 1080

tatatatgtc tactgttttt attgattcaa tatttgattg ttctttttt tgcagatttg 1140

ttgaccagag atctaccatg 1160

<210> SEQ ID NO 13
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(29)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 13

cccaagctta aatgacatca gatacacgc 29

<210> SEQ ID NO 14
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(24)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 14

cataagctta gaggtccaaa ttca 24

<210> SEQ ID NO 15
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(31)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 15

ccatcagcca tggtcttcta cctttatgca aa 32

<210> SEQ ID NO 16
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: artificial sequence

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<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(36)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 16

ccaagcttac cacactcaga tgcataaaca aacaca 36

<210> SEQ ID NO 17
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(31)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 17

catcagccat ggtctactct ctgcaaaaac a 31

<210> SEQ ID NO 18
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(27)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 18

gcaaagctta ctagtcaaca attggcc 27

<210> SEQ ID NO 19
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(28)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 19

gatcggccat ggttcactaa aaaaaaag 28

<210> SEQ ID NO 20
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(36)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 20

ggaagcttgc ggccgctttc tactctacat gtttct 36

<210> SEQ ID NO 21
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(31)
<223> OTHER INFORMATION: fully synthetic sequence

<400> SEQUENCE: 21

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gactagccgc catggttcaa tctctagctg a 31

<210> SEQ ID NO 22
<211> LENGTH: 1578
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1578)
<223> OTHER INFORMATION: Act1a promoter polynucleotide sequence and intron

<400> SEQUENCE: 22

taaatgacat cagatacacg cttgtgaacc atctttaaag tattgatgga ctcttcacta 60

tgaaagctct ctttaaaatt aattttcttt gtacatgtct ctaagcaatg tcaaattaat 120

tagaggtcca aattcaaaaa aatgtcgtat tgaatcattc cattactaaa ttggttcaat 180

gtcagattta aacagcctag ggataattta gtgagatatg agattctact ttcaacatat 240

actaatccta aatctctagc aacttttttat ataagctata aatatcatga aaatgtattt 300

taatcgtttc ataatttatg cagtcacact aatggaaaaa aggccaatta ttattatttt 360

cttcagacta taaatgaaaa cataaattaa aatgcagatt agtttaaaat tttaataagt 420

aagtaaaatg cttatagcct tatacaaaat catatttgga agtttctaac attgttgcaa 480

tttgttatca caaatcacag taatatttgt atactaatta gtaattacaa ctatacacaa 540

atttaaatgg gtaatcatat atttgtgtcc agtggattga acaaatatgc tcggcccatg 600

cggaagtaat gccaattttg ggtgagtaaa gcccatgcga aattttcaca taagaaatgc 660

atgctttttg ttttcaacga catgagttgc atgcttttta tcattgctta tatagttgca 720

agtttgcaac tccttgatat ttttttttatg tagacactac taccaccaaa aacttttggt 780

ctgcttattc ttgtttacta tgtaaaaaaa ataaatgaat tgtttattta ctccgatttg 840

atggagtctg gtttatgagg ttttatagcc tttacagaaa attgatagtt acaaaaatat 900

ttttcaaaaa taaaagggta aaaccgtcat ttcaagttgt tattgttttg ggggactgga 960

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cttcttcttc ccctctttca ttttccagcc actacaaact tttctatctc tacttttttt 1140

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gttcattaca ccatttgtta tttgtttaat caaccaaaag actcatgttt ttcaaatgtc 1380

tttaatataa tttttctgat tgaattttat aatatttaca tgattctgga tccagaatat 1440

ccttcttctt cttccatttt gtcctgtatt gatttgtctt tgaaaaagga ttgttctttg 1500

tatctgtatt ggtgaaaaag gattgttatt tgttgataaa aatttgatct ttaaacaatg 1560

tttggttttg cataaagg 1578

<210> SEQ ID NO 23
<211> LENGTH: 1468
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:

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<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1468)
<223> OTHER INFORMATION: Act1b promoter polynucleotide sequence and intron

<400> SEQUENCE: 23

ttagaggtcc aaattcaaaa aaatgtcgta ttgaatcatt ccattactaa attggttcaa 60

tgtcagattt aaacagccta gggataattt agtgagatat gagattctac tttcaacata 120

tactaatcct aaatctctag caacttttta tataagctat aaatatcatg aaaatgtatt 180

ttaatcgttt cataatttat gcagtcacac taatggaaaa aaggccaatt attattattt 240

tcttcagact ataaatgaaa acataaatta aaatgcagat tagtttaaaa ttttaataag 300

taagtaaaat gcttatagcc ttatacaaaa tcatatttgg aagtttctaa cattgttgca 360

atttgttatc acaaatcaca gtaatatttg tatactaatt agtaattaca actatacaca 420

aatttaaatg ggtaatcata tatttgtgtc cagtggattg aacaaatatg ctcggcccat 480

gcggaagtaa tgccaatttt gggtgagtaa agcccatgcg aaattttcac ataagaaatg 540

catgcttttt gttttcaacg acatgagttg catgcttttt atcattgctt atatagttgc 600

aagtttgcaa ctccttgata ttttttttat gtagacacta ctaccaccaa aaacttttgg 660

tctgcttatt cttgtttact atgtaaaaaa aataaatgaa ttgtttattt actccgattt 720

gatggagtct ggtttatgag gttttatagc ctttacagaa aattgatagt tacaaaaata 780

tttttcaaaa ataaaagggt aaaaccgtca tttcaagttg ttattgtttt gggggactgg 840

atttgaaatg aaatatagaa ccggaaaaca aggtgagccg aagtcgaagc ctttggaccc 900

gtttttatat ttactcctcc cattcccttc tccttcaatc cttccttcct cctcctccct 960

tcttcttctt cccctctttc attttccagc cactacaaac ttttctatct ctacttttt 1020

tcctctcgat ttcaggtact ttttgagacc ctttgttgtg attttcgaac acacacccca 1080

attacgtttg atttttgatc ccgcatcgat ttcaattcat ccgtttctga gtttcttttg 1140

gatctgggtg tcttgagcta atcttttcga tctgttgttt atcgatttta ctcatgcgta 1200

tgttcattac accatttgtt atttgtttaa tcaaccaaaa gactcatgtt tttcaaatgt 1260

ctttaatata atttttctga ttgaatttta taatatttac atgattctgg atccagaata 1320

tccttcttct tcttccattt tgtcctgtat tgatttgtct ttgaaaaagg attgttcttt 1380

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gtttggtttt gcataaaggt agaagacc 1468

<210> SEQ ID NO 24
<211> LENGTH: 1642
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1642)
<223> OTHER INFORMATION: Act3 promoter polynucleotide sequence and intron

<400> SEQUENCE: 24

tcaagcttac cacactcaga tgcataaaca aacacagcaa gaagattgcc acaaaaatca 60

taacgaaata atcaagagat agctatcaaa tcgccaccgg cgaatcatgt catactcagt 120

atcagaaaca gatatgatag ctcaaaatat ggattaataa tgttactaaa cacatggaca 180

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ataatgcatc aatattgaaa gaaagaaaat ggtttagcag aagcaaaatg gtttagaaag 240

taatgaacta cacattcaca aaggtgaaga attcgtcaag cctacaataa caaatgtcta 300

tactttatga gcccacaaag agatacatca cactatctga acgaaactaa agcaacctaa 360

catagtctag aaactactaa aatgaatgtt tcaaaacaat tttaacagaa ggcaaaagtg 420

aaacaacata ctcctttgcg agaacgagga cgaggagcta attcacgtct ggtaacaaca 480

tgtcccttgt tcaacccaac gaacaaaccg gtcttcactt gtggagttgt catcttctgt 540

aaatttcata gacaacaaac aaacaaaact ttctattcaa tacaaaatca aattttacaa 600

gagacggatt cagagataat aaagagatga agagagttaa atcaaaaggg attgatagaa 660

gatacctaat caatggatcg agctcctccg gtggttcaga caaaagaagg acgccgactg 720

aaaattacat ttttgtatat ataccagaga gactcaagaa aaaaccctag tccagtttgg 780

gcttttattg ggccttataa attttgggtc agttttgaca aagtaaatac aaggctatag 840

ctgctttgct aacgtgatta attatttacc atttaccaaa agccttaacc gaggccgagc 900

gagaaaaaaa aacaaaaaaa aggtagaggg caagaacgtc atttccacaa ggaattgaat 960

cggaaaacga ggtgtgccga attcgaagcc tttggacccg tttttatact tttttacttg 1020

ccattcgttt ttttttgttc attggcctca tttgattact tgtttctttg atttctcctt 1080

ccatagaacc gaattgtttt cagtctgaga tttctcctgc cgagagaacg attttaatct 1140

attttcctcg gtaatgttat agcctaattt gtgtttttt cttttcctg atccggatat 1200

cgttattctg attgacaatt gtcagtttca tcttctattc tgtgaaattt tgatttttt 1260

ccgatctgtg atttcgtcat tgtatcagcg tgcttatatg cgtttgaggc gtaaatgagt 1320

gtgtacctca tttatcattt gctatgtttt ttttttaac agagatcttc agctgtaata 1380

ttataataga ttgaattgat aacgtgattc tggatctgga atatatatat gtcacattct 1440

tcttaggatt tgattttgtc tctctttgga tattaatatt cttcactccc ttgaaaatga 1500

atctgtttat tataatgttt agatatattc cttaccggca tttgttttag cataaatatg 1560

aaacatagca ttgactgatt tgtctttta ttattcttgt ttttttgcca aattggtctc 1620

atgtttttgc agagagtaga cc 1642

<210> SEQ ID NO 25
<211> LENGTH: 1241
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1241)
<223> OTHER INFORMATION: n= a or g or c or t/u
Act7 promoter polynucleotide sequence and intron

<400> SEQUENCE: 25

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attctccttg gttgcaacag tctacccgtc aaatgtttac taatttataa gtgtgaagtt 120

tgaattatga aaaacgaaat cgtattaaaa attcacaaga ataaacaact ccatagattt 180

tcaaaaaaac agtcacgaga aaaaaaccac agaccgtttg tctgctcttc tagtttttat 240

tatttttcta ttaatagttt tttgttattt cgagaataaa atttgaacga tgtccgaacc 300

acaaaagccg agccgataaa tcctaagccg agcctaactt tagccgtaac catcagtcac 360

ggctcccggg ctaattcatt tgaaccgaat cataatcaac ggtttagatc aaactcaaaa 420

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caatctaacg gcaacataga cgcgtcggtg agctaaaaag agtgtgaaag ccaggtcacc 480

atagcattgt ctctcccaga ttttttattt gggaaataat agaagaaata gaaaaaaata 540

aaagagtgag aaaaatcgta gagctatata ttcgcacatg tactcgtttc gctttcctta 600

gtgttagctg ctgccgctgt tgtttctcct ccatttctct atctttctct ctcgctgctt 660

ctcgaatctt ctgtatcatc ttcttcttct tcaaggtgag tctctagatc cgttcgcttg 720

attttgctgc tcgttagtcg ttattgttga ttctctatgc cgatttcgct agatctgttt 780

agcatgcgtt gtggttttat gagaaaatct ttgttttggg ggttgcttgt tatgtgattc 840

gatccgtgct tgttggatcg atctgagcta attcttaagg tttatgtgtt agatctatgg 900

agtttgagga ttcttctcgc ttctgtcgat ctctcgctgt tatttttgtt tttttcagtg 960

aagtgaagtt gtttagttcg aaatgacttc gtgtatgctc gattgatctg gttttaatct 1020

tcgatctgtt aggtgttgat gtttacaagt gaattctagt gttttctctt tgagatctgt 1080

gaagtttgaa cctagttttc tcaataatca acatatgaag cgatgtttga gtttcaataa 1140

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caattcattt cggtttcatt ttacttttt tttagtgaac c 1241

<210> SEQ ID NO 26
<211> LENGTH: 1313
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1313)
<223> OTHER INFORMATION: polynucleotide sequence of Act12 promoter and intron

<400> SEQUENCE: 26

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tcaacatgac aaaatgaatg aatgtttgct ttaattggtg gctaaaagct aaatacacag 180

aaaagtcaaa attcaatctc aaaatcaacc cctctgtctc caatgtccct aatctatacc 240

aaaatgtcaa tttatttttct tgatcatata ttccactaat taaaaataaa tccttctcta 300

atgaaatttg tcaaggcctt ggaagcctag ttttaaatat taaatggaaa ctatttcttc 360

aacaatcaca ctgttattta gtattgttgt atgttgttca ctactttctt catttgtttt 420

gtaagaaact ataataagca aaaacacata ataaagtctc atgtcaaata atgaatctta 480

tgcacatgct tgattatttt acttgcacat atccctatca tcattatcac atttgtcaat 540

taccgttatc atcattactc tcattcttcc cagaactttt tcagcaattt ccatacctca 600

cccactaaga tcttttaccc tttttcttaa ttatagtttg gatagcactc ttttacatag 660

cactgaaatt tcggttgaac acataaatta ctagaaacta gaaggaaatg ttactgaaat 720

ttcactgatt gtctaaaatt gaataatcta aagaaaatgg ccttttaacc tttttcttag 780

gcccaaatgg gctcattacc actcatgctt gttcggtgac ccgattcttc cggtaaaaca 840

gagcctaaac cgtattttca ggttaggctg gtgttttctt aattctccaa cctaaaaata 900

gatggacacg tgtctataga ggctgagata ttggtctcaa tgaagaaaac taacggctca 960

gacccgtgta tgaacgatat taagggccaa agttgcttct gttttccaga aatttttgaa 1020

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acccaatttc agggcacgat tccacaacct ctttcttttc ttctagatct acgtaaattc 1080

atcaggtaca tgttattttt tttgtttatt tgatgtcaaa attttgatca caaggaggca 1140

aaaccaatat aaatgtaacg ctaatgcgtt tgattatggt atacgtaacg aattagattt 1200

aatggttaca ttttattgtt ttagatttag ttatgagatt ggcattaatt attggtgttt 1260

cctttgaatt tgctatgttt cttatgttga tgtaatcagc tagagattga acc 1313

<210> SEQ ID NO 27
<211> LENGTH: 1946
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1946)
<223> OTHER INFORMATION: chimeric promoter fusion FMV and Act11 polynucleotides + Act11 intro

<400> SEQUENCE: 27

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tcatggtcag taagtttcag aaaaagacat ccaccgaaga cttaaagtta gtgggcatct 180

ttgaaagtaa tcttgtcaac atcgagcagc tggcttgtgg ggaccagaca aaaaaggaat 240

ggtgcagaat tgttaggcgc acctaccaaa agcatctttg cctttattgc aaagataaag 300

cagattcctc tagtacaagt ggggaacaaa ataacgtgga aaagagctgt cctgacagcc 360

cactcactaa tgcgtatgac gaacgcagtg acgaccacaa aagaattagc ttgagctcag 420

gatttagcag cattccagat tgggttcaat caacaaggta cgagccatat cactttattc 480

aaattggtat cgccaaaacc aagaaggaac tcccatcctc aaaggtttgt aaggaagaat 540

tcgatatccc cgcggccgcg ttatcacaac caaatgtcaa atggaatgca tcagagacca 600

aacctgtaag agtccacaaa acaattcaaa gaaagaatat caacaattca gagattcaat 660

cctaaaacaa aaagagaact gaaaccaaat cgtacctaca cgaccagtga agataccaat 720

agagagctct gttgtagaat acaacacatt aagcgcaatt agcagaaaca gtctcttcat 780

ctgccgattt ccacttgtca ctactccaaa aacctcccaa accatttcca aaacagacac 840

ttttgccatg tctacatctt tcccttcccc gaaaaacaca tcatttccat caacggagta 900

aatatccggc ggcatatcga tgctcgagac cgtcctatcg agaaaaggct tagccgcttc 960

cgtgaccgcc ggcgttcgtg gaccgtgaga ttgctgaaac gagcgagaat aagcaagcct 1020

ccgatcatta gcagcatatc cgacatcgct gctccgatca tcagggagct cgttatcgcc 1080

tcgaggatta aaggaaatgg atctctccat tttcttcttt gatcttaaag ttccaacttc 1140

ggcaaatact aaaatcaaca gtcagtcgta caaagaaact ctgcttatac agtaaagtca 1200

atgggccact gttctaagcc catatataat tttagaagcc catagaatac aaaagagtca 1260

agaagcattg accgcacaag aaaaaaacaa ttgttaaaaa gggttggtta gtgtgtatgt 1320

atatatgaaa tgcaacaaac attatacagc ccattaaata tggttgttat aggtagatgt 1380

ccccattaag gaactttatc cagcccatta aattacttta cagagtaaaa gagagagaga 1440

agatttacag ttacgttacc aaattttcga aatgatttaa ttagtaataa ataaataatt 1500

aaatgtcagt tactctcttt agaaagctaa ataagacagc tgtttccacc aacaacgtga 1560

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ctggtcgtgg ggtcctcctt cgttcaaagt gatattcaga aatcaacggc tgagatcttc 1620

tccatcaata tttattacgg gcctattcct tccttttta aacttcaatt ctccggctca 1680

cattctcttc ttcattcgct ccgtttctct ctcaaaaact acacacccgt accacaccac 1740

caccctcctc gtttcctcag agatcccctc tctaacttct aaggtaatca catttccata 1800

acgttccatc gtcattgatt cttcattagt atgcgtttat gaagcttttt caatttaatt 1860

ctctttggta gatcttaaga ttcctctgtt tcttgcaaaa taaagggttc aattatgcta 1920

atatttttta tatcaatttt gacagg 1946

<210> SEQ ID NO 28
<211> LENGTH: 1695
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1695)
<223> OTHER INFORMATION: n= a or g or c or t/u, r = g or a, y = t/u or c
chimeric promoter fusion FMV and EF1 polynucleotides + EF1 intro

<400> SEQUENCE: 28

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agctacagga gatcaatgaa gaatcttcaa tcaaagtaaa ctactgttcc agcacatgca 120

tcatggtcag taagtttcag aaaaagacat ccaccgaaga cttaaagtta gtgggcatct 180

ttgaaagtaa tcttgtcaac atcgagcagc tggcttgtgg ggaccagaca aaaaaggaat 240

ggtgcagaat tgttaggcgc acctaccaaa agcatctttg cctttattgc aaagataaag 300

cagattcctc tagtacaagt ggggaacaaa ataacgtgga aaagagctgt cctgacagcc 360

cactcactaa tgcgtatgac gaacgcagtg acgaccacaa aagaattagc ttgagctcag 420

gatttagcag cattccagat tgggttcaat caacaaggta cgagccatat cactttattc 480

aaattggtat cgccaaaacc aagaaggaac tcccatcctc aaaggtttgt aaggaagaat 540

tcgatatcaa gcttgatatc ggaagtttct ctcttgaggg aggttgctcg tggaatggga 600

cacatatggt tgttataata aaccatttcc attgtcatga gattttgagg ttaatatata 660

ctttacttgt tcattatttt atttggtgtt tgaataaatg atataaatgg ctcttgataa 720

tctgcattca ttgagatatc aaatatttac tctagagaag agtgtcatat agattgatgg 780

tccacaatca atgaaatttt tgggagacga acatgtataa ccatttgctt gaataacctt 840

aattaaaagg tgtgattaaa tgatgtttgt aacatgtagt actaaacatt cataaaacac 900

aaccaaccca agaggtattg agtattcacg gctaaacagg ggcataatgg taatttaaag 960

aatgatatta ttttatgtta aaccctaaca ttggtttcgg attcaacgct ataaataaaa 1020

ccactctcgt tgctgattcc atttatcgtt cttattgacc ctagccgcta cacacttttc 1080

tgcgatatct ctgaggtaag cgttaacgta cccttaratc gttcytttte yttttcgtct 1140

gctgatcgtt gctcatatta tttcgatgat tgttggattc gatgctcttt gttgattnat 1200

cgttctgaaa attctnatct gttgtttaga ttttatcgat tgttaatatc aacgtttcac 1260

tgcttctaaa cgataattta ttcatgaaac tattttccca ttctgatcga tcttgttttg 1320

agattttaat ttgttcgatt gattgttggt tggtggatct atatacgagt gaacttgttg 1380

atttgcgtat ttaagatgta tgtcgatttg aattgtgatt gggtaattct ggagtagcat 1440

aacaaatcca gtgttccctt tttctaaggg taattctcgg attgtttgct ttatatctct 1500

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tgaaattgcc gatttgattg aatttagctc gcttagctca gatgatagag caccacaatt 1560

tttgtggtag aaatcggttt gactccgata gcggcttttt actatgattg ttttgtgtta 1620

aagatgattt tcataatggt tatatatgtc tactgttttt attgattcaa tatttgattg 1680

ttctttttt tgcag 1695

<210> SEQ ID NO 29
<211> LENGTH: 1800
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1800)
<223> OTHER INFORMATION: y = t/u or c
chimeric promoter fusion CaMV and Act8 polynucleotides + Act8 intro

<400> SEQUENCE: 29

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cccagctatc tgtcacttta ttgtgaagat agtggaaaag gaaggtggct cctacaaatg 120

ccatcattgc gataaaggaa aggccatcgt tgaagatgcc tctgccgaca gtggtcccaa 180

agatggaccc ccacccacga ggagcatcgt ggaaaaagaa gacgttccaa ccacgtcttc 240

aaagcaagtg gattgatgtg atggtccgat gtgagacttt tcaacaaagg gtaatatccg 300

gaaacctcct cggattccat tgcccagcta tctgtcactt tattgtgaag atagtggaaa 360

aggaaggtgg ctcctacaaa tgccatcatt gcgataaagg aaaggccatc gttgaagatg 420

cctctgccga cagtggtccc aaagatggac ccccacccac gaggagcatc gtggaaaaag 480

aagacgttcc aaccacgtct tcaaagcaag tggattgatg tgatatcaag cttccatttt 540

tcttttgcat aattcatgtt tatttttta ttttttcat cttgcataat tcatgtttaa 600

aaggatatat acatgggtct actacattct cctgacatta cgtttatgt gtttgtcttc 660

tgaaaataat catcaaaata tttcaggact tgtttacgtt ttcaggagaa aaaaaataac 720

tgtacccttt tcaatataga aataacattt gtagaaatcg tggattttcc ttaataaaca 780

atccaaaaca cgaccaccgt tgtctcctcg actcggtaac acccgatcgc cgacttgaaa 840

attagaagaa aaatgaaaag aataataaaa aaaaaaaagg aatgatgatt gaagctgtca 900

tatatgtcga ccctatcaca gtcaatccaa tagcctatat tcgccaactg atatatccaa 960

cggctcacaa attttcacaa acttttcaaa aaagtataat aaaagaggct gtctgacagc 1020

catgtcacgt tatacttttt ccgtatgatc gaaatgattc gtctttgyyg aatttaatta 1080

tttccaaaat tgaygactct aaagaaaaaa aaatagtttt tcagataaac ccgcctatat 1140

aaatagttca acactcggtt tatttcttct cccctcaaag aattgcctcg tcgtcttcag 1200

cttcatcggc cgttgcattt cccggcgata agagagagaa agaggagaaa gagtgagcca 1260

gatcttcatc gtcgtggttc ttgtttcttc ctcgatctct cgatcttctg cttttgcttt 1320

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tcttgcgttg tatttgaaat cgatccggat cctttcgctt cttctgtcaa taggaatctg 1560

aaatttgaaa tgttagttga agtttgactt cagattctgt tgatttattg actgtaacat 1620

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atacatcata cattgaattg aatctactca tgaatgaaaa gcctggtttg attaagaaag 1740

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<210> SEQ ID NO 30
<211> LENGTH: 1742
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1742)
<223> OTHER INFORMATION: chimeric promoter fusion CaMV and Act2
polynucleotides + Act2 intro

<400> SEQUENCE: 30

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ccagctatct gtcactttat tgtgaagata gtggaaaagg aaggtggctc ctacaaatgc 120

catcattgcg ataaaggaaa ggccatcgtt gaagatgcct ctgccgacag tggtcccaaa 180

gatggacccc cacccacgag gagcatcgtg gaaaaagaag acgttccaac cacgtcttca 240

aagcaagtgg attgatgtga tggtccgatg tgagactttt caacaaaggg taatatccgg 300

aaacctcctc ggattccatt gcccagctat ctgtcacttt attgtgaaga tagtggaaaa 360

ggaaggtggc tcctacaaat gccatcattg cgataaagga aaggccatcg ttgaagatgc 420

ctctgccgac agtggtccca aagatggacc cccacccacg aggagcatcg tggaaaaaga 480

agacgttcca accacgtctt caaagcaagt ggattgatgt gatatcaagc ttcaactatt 540

tttatgtatg caagagtcag catatgtata attgattcag aatcgttttg acgagttcgg 600

atgtagtagt agccattatt taatgtacat actaatcgtg aatagtgata tgatgaaaca 660

ttgtatctta ttgtataaat atccataaac acatcatgaa agacactttc tttcacggtc 720

tgaattaatt atgatacaat tctaatagaa aacgaattaa attacgttga attgtatgaa 780

atctaattga acaagccaac cacgacgacg actaacgttg cctggattga ctcggtttaa 840

gttaaccact aaaaaaacgg agctgtcatg taacacgcgg atcgagcagg tcacagtcat 900

gaagccatca aagcaaaaga actaatccaa gggctgagat gattaattag tttaaaaatt 960

agttaacacg agggaaaagg ctgtctgaca gccaggtcac gttatcttta cctgtggtcg 1020

aaatgattcg tgtctgtcga ttttaattat ttttttgaaa ggccgaaaat aaagttgtaa 1080

gagataaacc cgcctatata aattcatata ttttcctctc cgctttgaat tgtctcgttg 1140

tcctcctcac tttcatcagc cgttttgaat ctccggcgac ttgacagaga agaacaagga 1200

agaagactaa gagagaaagt aagagataat ccaggagatt cattctccgt tttgaatctt 1260

cctcaatctc atcttcttcc gctctttctt tccaaggtaa taggaacttt ctggatctac 1320

tttatttgct ggatctcgat cttgttttct caatttcctt gagatctgga attcgtttaa 1380

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ttcatgttac ctgggaaatg atttgtatat gtgaattgaa atctgaactg ttgaagttag 1560

attgaatctg aacactgtca atgttagatt gaatctgaac actgtttaag ttagatgaag 1620

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tttgtgtata gattcttcga aactttagga tttgtagtgt cgtacgttga acagaaagct   1680

atttctgatt caatcagggt ttatttgact gtattgaact ctttttgtgt gtttgcagca   1740

ga                                                                  1742
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We claim:

1. A DNA construct comprising: an expression cassette comprising a promoter DNA sequence comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; a structural DNA sequence that encodes an agronomically useful protein; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter. DNA sequence is heterologous with respect to the structural DNA sequence.

2. A DNA construct of claim 1, wherein the structural DNA sequence encodes a herbicide tolerance gene.

3. A DNA construct of claim 2, wherein the herbicide tolerance gene is a glyphosate tolerance gene.

4. A DNA construct of claim 3, wherein the glyphosate tolerance gene is an EPSP synthase gene or a glyphosate oxidoreductase gene.

5. A DNA construct of claim 4, wherein the glyphosate tolerance gene is an aroA:CP4 gene.

6. A DNA construct of claim 3, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 50 grams acid equivalent per liter of glyphosate.

7. A DNA construct of claim 6, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 300 grams acid equivalent per liter of glyphosate.

8. A DNA construct of claim 3, that, when introduced into a plant cell, confers to the plant cell tolerance to at least one application of glyphosate at a rate of 16 oz per acre.

9. A DNA construct of claim 1, wherein the structural DNA sequence is a *Bacillus thuringiensis* insect control gene.

10. A DNA construct of claim 3, wherein the promoter DNA sequence consists essentially of a 5' regulatory region derived from a sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, and SEQ ID NO: 26.

11. A chimeric promoter DNA sequence comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; and said at least one cis element is operably linked to a heterologous promoter sequence.

12. A chimeric promoter DNA sequence of claim 11, wherein the heterologous promoter sequence is a plant DNA virus promoter.

13. A chimeric promoter DNA sequence of claim 12 wherein the plant DNA virus promoter is selected from the group consisting of Cauliflower mosaic virus and Figwort mosaic virus.

14. A chimeric promoter DNA sequence of claim 13, wherein the chimeric promoter DNA sequence is selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30.

15. A DNA construct comprising: and expression cassette comprising a chimeric promoter DNA sequence comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; and said at least one cis element is operably linked to a heterologous promoter sequence; a structural DNA sequence; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter DNA sequence is heterologous with respect to the structural DNA sequence.

16. A DNA construct of claim 15, wherein the structural DNA sequence encodes a herbicide tolerance gene.

17. A DNA construct of claim 16, wherein the herbicide tolerance gene is a glyphosate tolerance gene.

18. A DNA construct of claim 17, wherein the glyphosate tolerance gene is an EPSP synthase gene or a glyphosate oxidoreductase gene.

19. A DNA construct of claim 17, wherein the glyphosate tolerance gene is an aroA:CP4 gene.

20. A DNA construct of claim 17, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 50 grams acid equivalent per liter of glyphosate.

21. A DNA construct of claim 17, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 300 grams acid equivalent per liter of glyphosate.

22. A DNA construct of claim 17, that, when introduced into a plant cell, confers to the plant cell tolerance to at least one application of glyphosate at a rate of 16 oz per acre.

23. A DNA construct of claim 15, wherein the structural DNA sequence is an insect control gene.

24. A DNA construct of claim 23, wherein the insect control gene is derived from *Bacillus thuringieneis.*

25. A DNA construct comprising a chimeric promoter DNA sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; a structural DNA sequence; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the chimeric promoter and the 3' non-translated region, and

the chimeric promoter DNA sequence is heterologous with respect to the structural DNA sequence.

26. A DNA construct of claim 25, wherein the structural DNA sequence encodes a herbicide tolerance gene.

27. A DNA construct of claim 26, wherein the herbicide tolerance gene is a glyphosate tolerance gene.

28. A DNA construct of claim 27, wherein the glyphosate tolerance gene is an EPSP synthase gene or a glyphosate oxidoreductase gene.

29. A DNA construct of claim 27, wherein the glyphosate tolerance gene is an aroA:CP4 gene.

30. A DNA construct of claim 27, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 50 grams acid equivalent per liter of glyphosate.

31. A DNA construct of claim 27, that, when introduced into a plant cell, confers to the plant cell tolerance to an aqueous glyphosate formulation comprising at least 300 grams acid equivalent per liter of glyphosate.

32. A DNA construct of claim 27, that, when introduced into a plant cell, confers to the plant cell tolerance to at least one application of glyphosate formulation at a rate of 16 oz per acre.

33. A transgenic crop plant comprising the DNA construct of claim 2.

34. A transgenic crop plant of claim 33, wherein said crop plant is a monocot crop species.

35. A transgenic crop plant of claim 33, wherein said crop plant is a dicot crop species.

36. A transgenic crop plant of claim 33, wherein the herbicide tolerance gene is a glyphosate tolerance gene.

37. A transgenic crop plant comprising the DNA construct of claim 9.

38. A transgenic crop plant of claim 37, wherein said crop plant is a monocot crop species.

39. A transgenic crop plant of claim 37, wherein said crop plant is a dicot crop species.

40. A transgenic crop plant comprising the DNA construct of claim 15.

41. A transgenic crop plant of claim 40, wherein said crop plant is a monocot crop species.

42. A transgenic crop plant of claim 40, wherein said crop plant is a dicot crop species.

43. A transgenic crop plant comprising the DNA construct of claim 25.

44. A transgenic crop plant of claim 43, wherein said crop plant is a monocot crop species.

45. A transgenic crop plant of claim 43, wherein said crop plant is a dicot crop species.

46. A DNA construct comprising a first expression cassette and a second expression cassette, wherein said first expression cassette comprises a promoter DNA sequence comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; a structural DNA sequence that encodes an agronomically useful protein; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter DNA sequence is heterologous with respect to the structural DNA sequence; and said second expression cassette comprises a promoter DNA sequence comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; a structural DNA sequence that encodes an agronomically useful protein; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter DNA sequence is heterologous with respect to the structural DNA sequence.

47. A method of expressing a structural DNA sequence in a plant, the method comprising: (1) providing a DNA construct comprising a promoter that is functional in a plant cell, the promoter comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12;, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; a structural DNA sequence encoding an agronomically useful protein; and a 3' non-translated region that functions to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter is heterologous with respect to the structural DNA sequence; (2) introducing the DNA construct into a plant cell; and (3) regenerating the plant cell to produce the plant such that the structural DNA sequence is expressible in the plant.

48. A method of expressing a structural DNA sequence in a plant, the method comprising: (1) providing a DNA construct comprising a promoter that is functional in a plant cell, the promoter comprising a sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; a structural DNA sequence; and a 3' non-translated region that functions to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the structural gene is operably linked to the promoter and the 3' non-translated region, and the promoter is heterologous with respect to the structural DNA sequence; (2) introducing the DNA construct into a plant cell; and (3) regenerating the plant cell to produce the plant such that the structural DNA sequence is expressible in the plant.

49. A method of controlling weeds, the method comprising: (1) providing a crop plant transformed with a DNA construct that comprises a promoter that is functional in a plant cell, the promoter comprising at least one cis element derived from a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12;, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26; a glyphosate tolerance gene; and a 3' non-translated region that functions to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the glyphosate tolerance gene is operably linked to the promoter and the 3' non-translated region, and the promoter is heterologous with respect to the glyphosate tolerance gene; and (2) applying to the crop plant a sufficient amount of glyphosate to control weeds without damaging the crop plant.

50. A method of controlling weeds, the method comprising: (1) providing a crop plant transformed with a DNA construct that comprises a promoter that is functional in a

plant cell, the promoter comprising a sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29; and SEQ ID NO:30; a glyphosate tolerance gene; and a 3' non-translated region that functions to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence; wherein the glyphosate tolerance gene is operably linked to the promoter and the 3' non-translated region, and the promoter is heterologous with respect to the glyphosate tolerance gene; and (2) applying to the crop plant a sufficient amount of glyphosate to control weeds without damaging the crop plant.

* * * * *

US006462258B1

(12) **United States Patent**
Fincher et al.

(10) **Patent No.: US 6,462,258 B1**
(45) **Date of Patent: Oct. 8, 2002**

(54) **PLANT EXPRESSION CONSTRUCTS**

(75) Inventors: **Karen L. Fincher**, Pacific, MO (US); **Jack Q. Wilkinson**, Redwood City, CA (US)

(73) Assignee: **Monsanto Technology LLC**, St. Louis, MO (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 87 days.

(21) Appl. No.: **09/737,698**

(22) Filed: **Dec. 15, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/171,173, filed on Dec. 16, 1999.

(51) **Int. Cl.**[7] **A01H 1/00**; A01H 1/06; A01H 4/00; C12N 15/82

(52) **U.S. Cl.** **800/300**; 435/320.1; 435/410; 435/413; 536/23.1; 536/23.2; 536/23.6; 536/24.1; 800/278

(58) **Field of Search** 435/69.1, 91.4, 435/235.1, 320.1, 325, 366, 455, 456, 457, 468, 418; 800/278, 205, 300; 536/23.2, 23.6, 24.1

(56) **References Cited**

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5,633,435 A * 5/1997 Barry et al. 800/205

* cited by examiner

Primary Examiner—David Guzo
Assistant Examiner—Maria B. Marvich
(74) *Attorney, Agent, or Firm*—Grace L. Bonner; Howrey Simon Arnold & White

(57) **ABSTRACT**

The present invention relates to a novel plant expression construct. More specifically the present invention provides a DNA construct comprising 5' regulatory sequences for modulating the expression of operably linked genes in plants.

5 Claims, 18 Drawing Sheets

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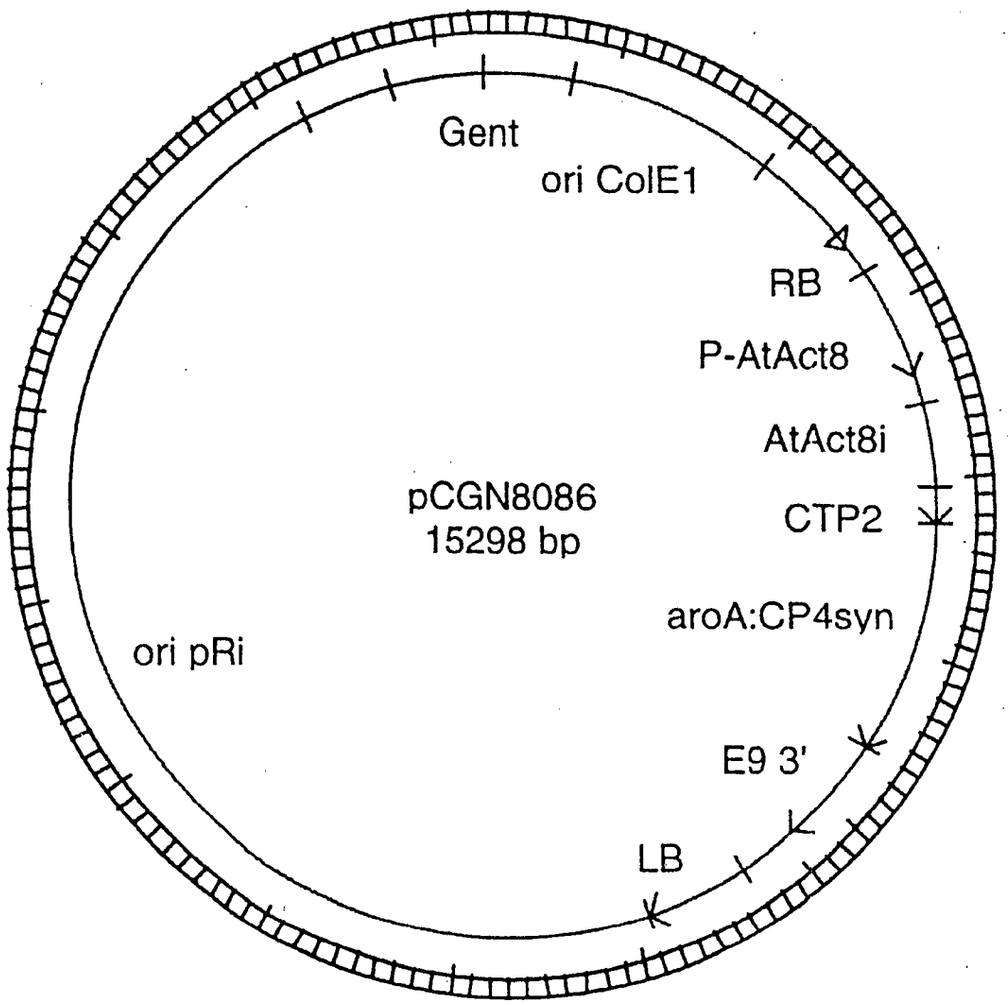

Gent
ori ColE1
RB
P-AtAct8
AtAct8i
CTP2
pCGN8086
15298 bp
aroA:CP4syn
ori pRi
E9 3'
LB

Figure 1

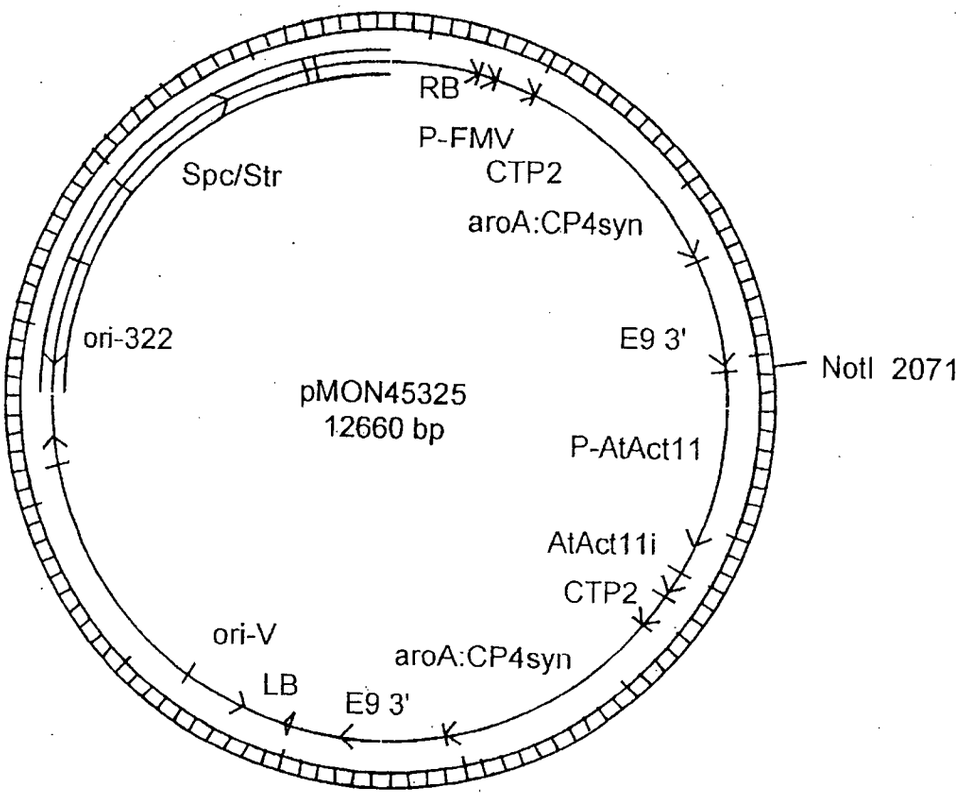


FIG. 2

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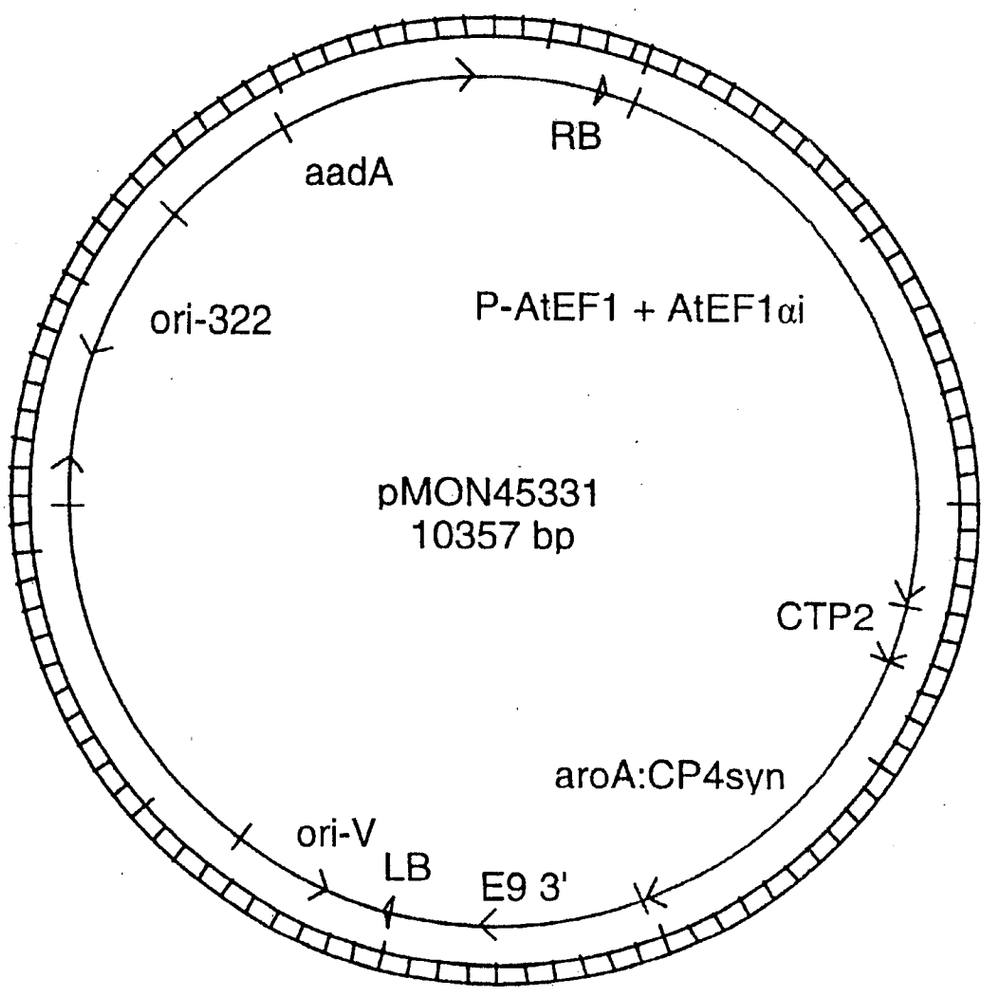

RB
aadA
ori-322
P-AtEF1 + AtEF1αi
pMON45331
10357 bp
CTP2
aroA:CP4syn
ori-V
LB
E9 3'

Figure 3

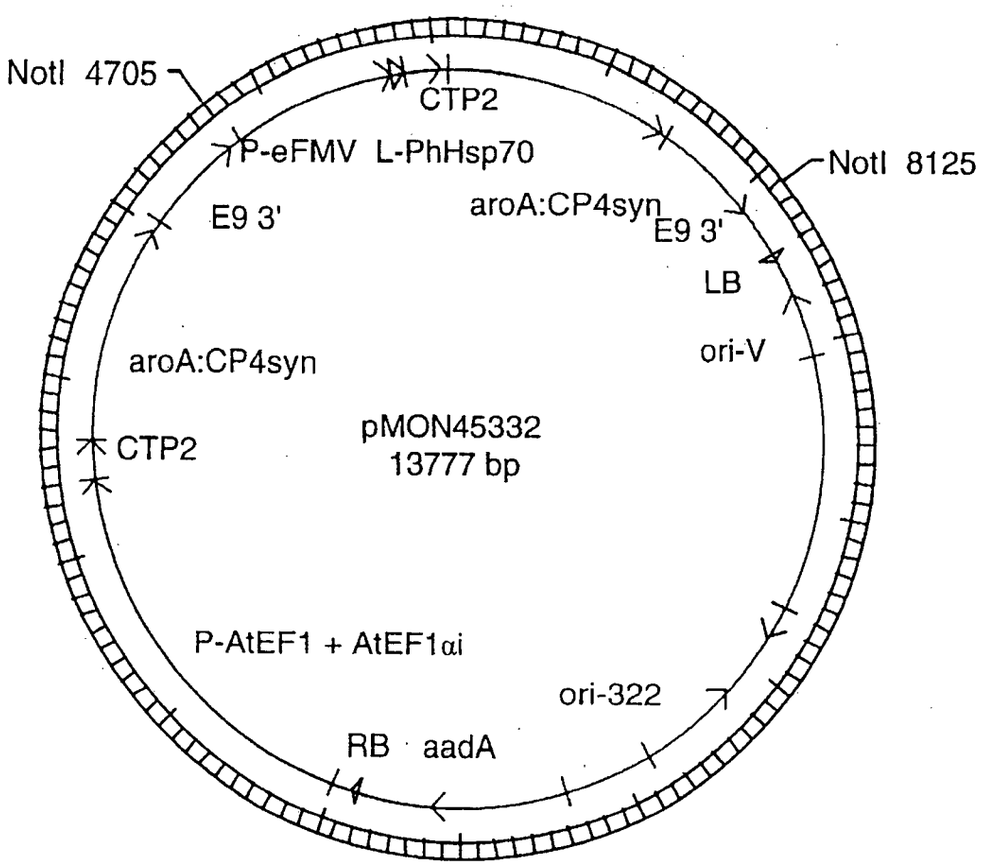


Figure 4

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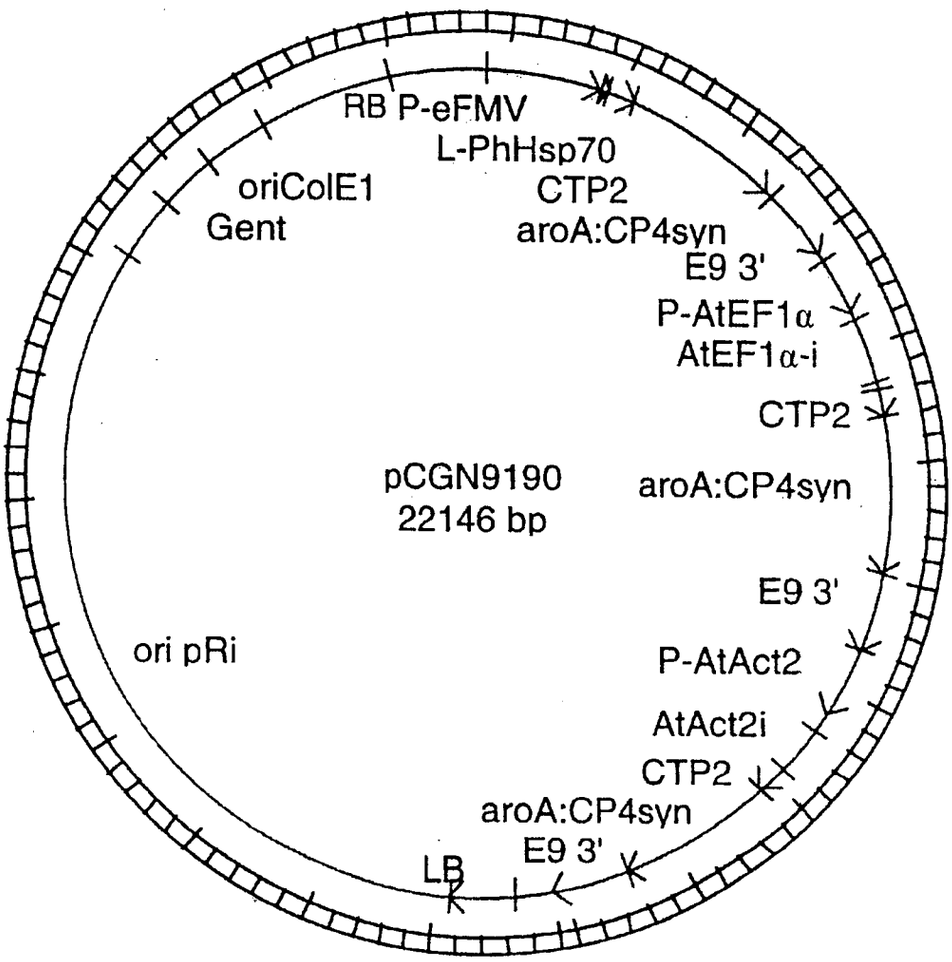

RB
P-eFMV
L-PhHsp70
CTP2
aroA:CP4syn
E9 3'
P-AtEF1α
AtEF1α-i
CTP2
aroA:CP4syn
E9 3'
P-AtAct2
AtAct2i
CTP2
aroA:CP4syn
E9 3'
LB
ori pRi
oriColE1
Gent
pCGN9190
22146 bp

Figure 5

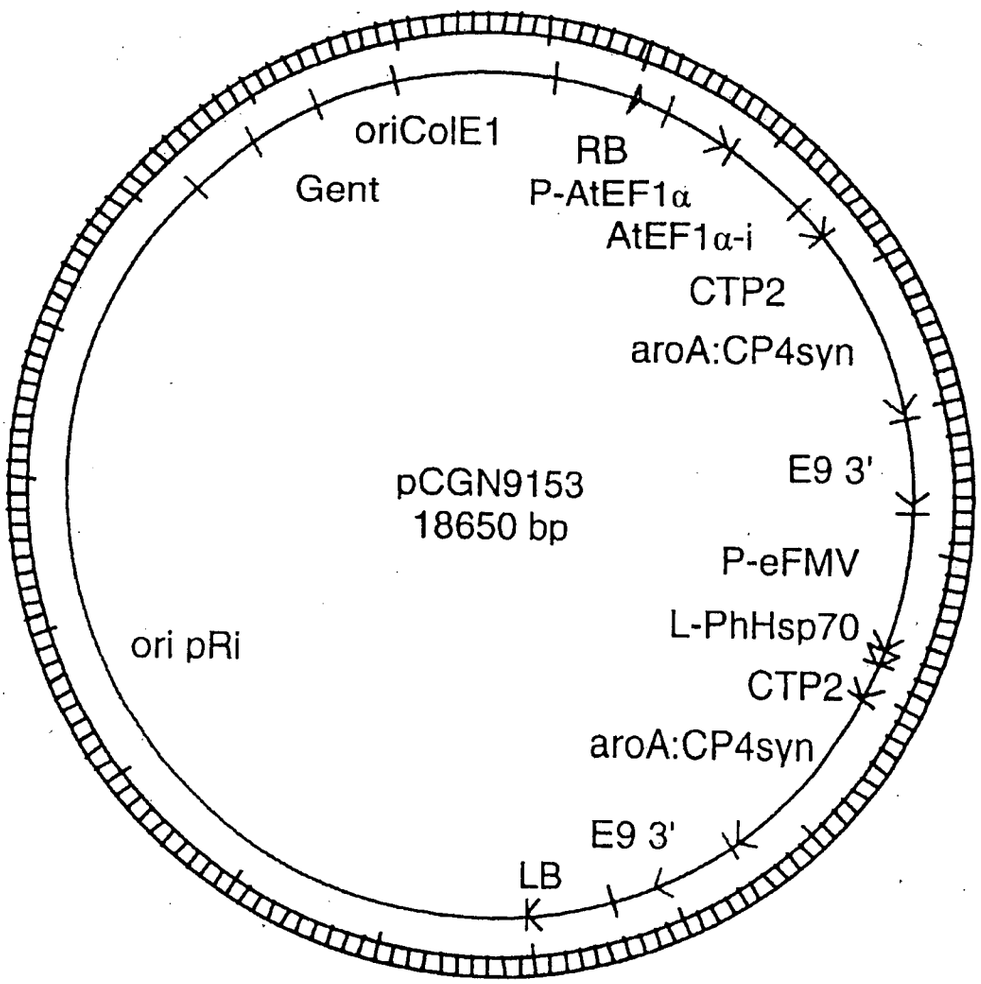


Figure 6

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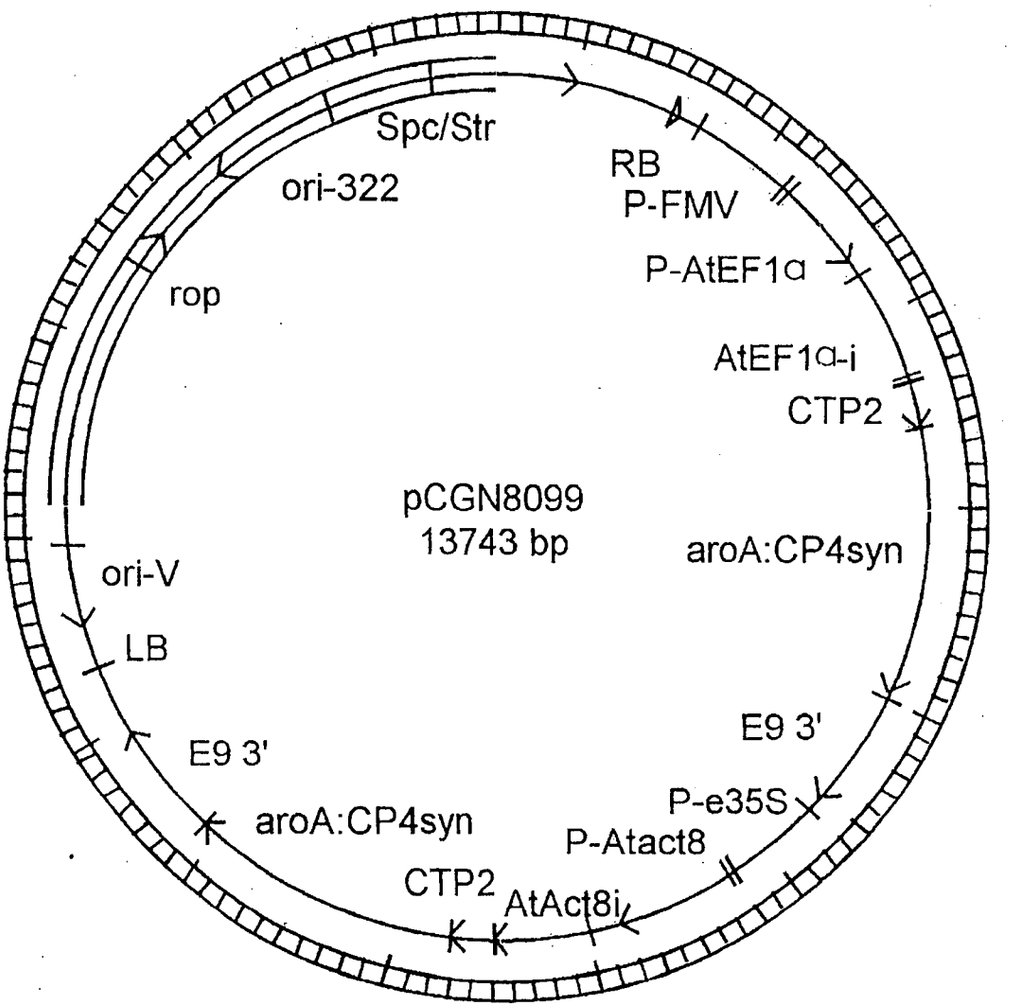

Spc/Str
ori-322
rop
RB
P-FMV
P-AtEF1α
AtEF1α-i
CTP2
pCGN8099
13743 bp
aroA:CP4syn
ori-V
LB
E9 3'
aroA:CP4syn
CTP2
AtAct8i
P-Atact8
P-e35S
E9 3'

FIG. 7

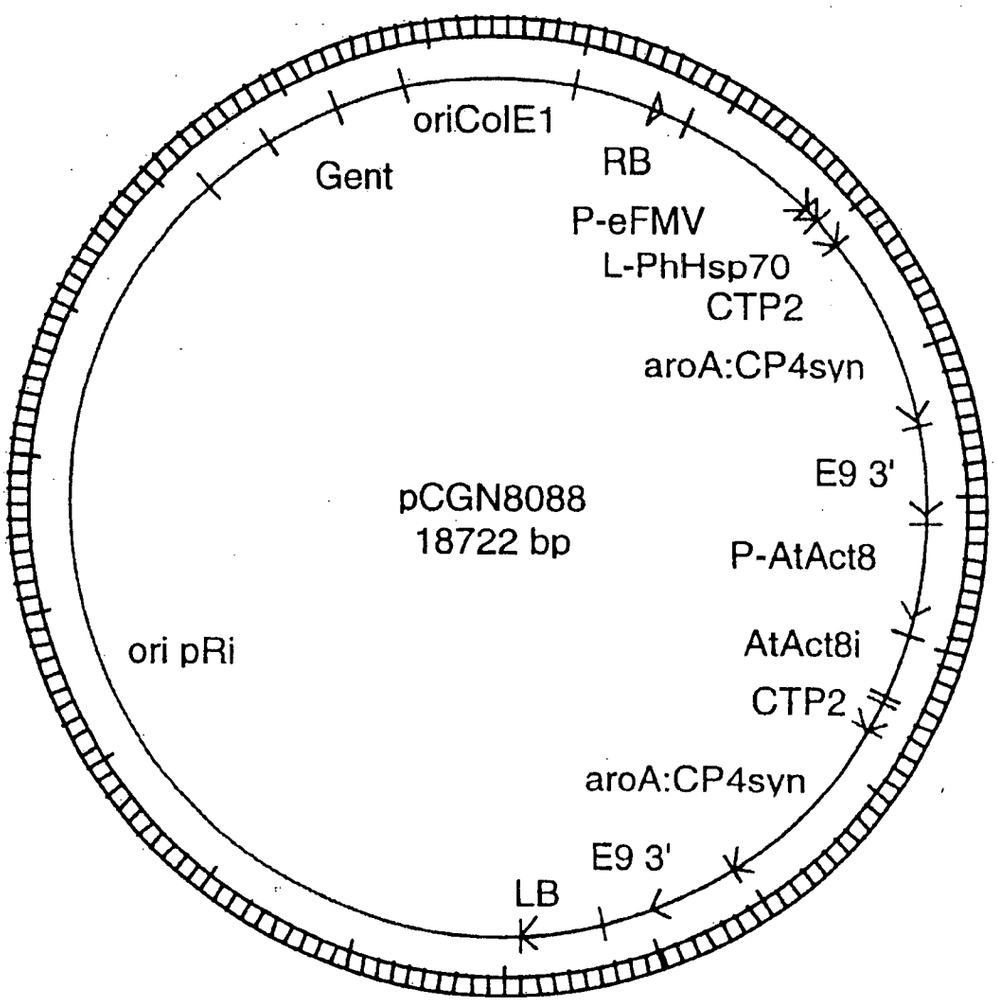


Figure 8

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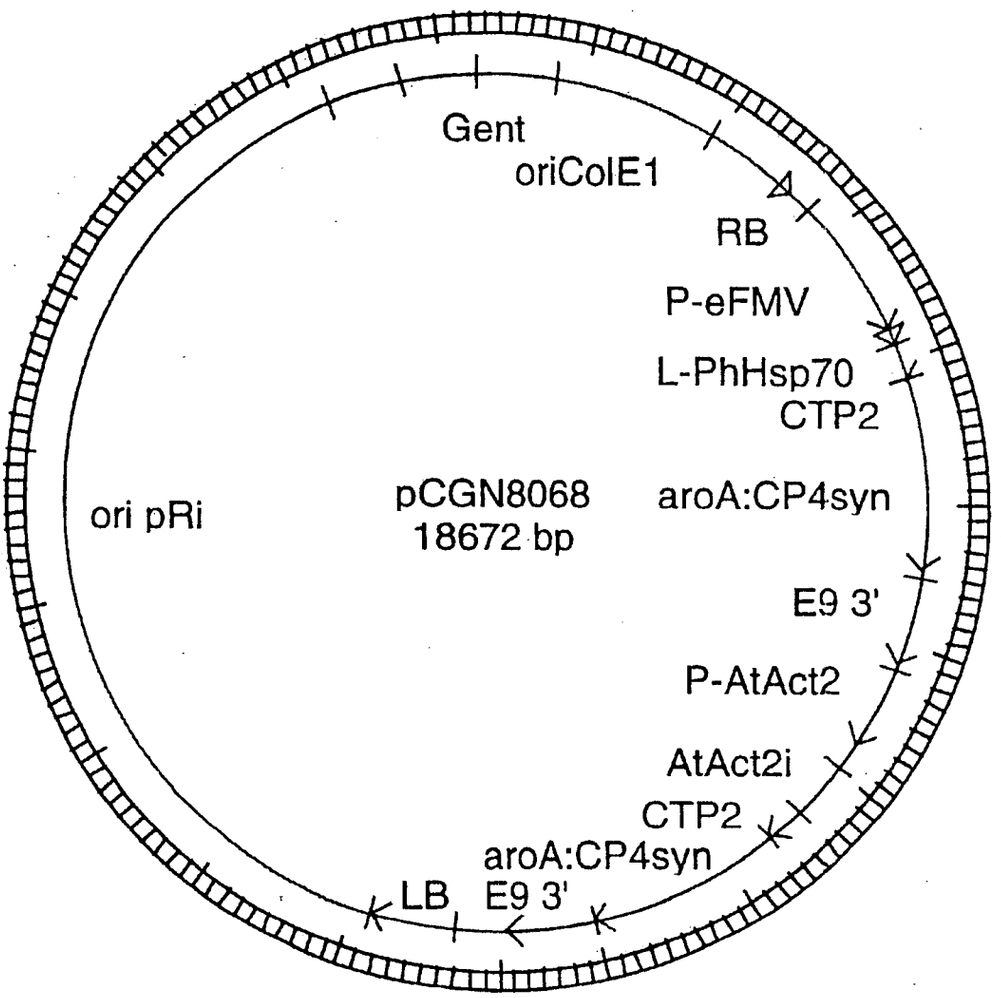

Gent
oriColE1
RB
P-eFMV
L-PhHsp70
CTP2
pCGN8068
18672 bp
aroA:CP4syn
ori pRi
E9 3'
P-AtAct2
AtAct2i
CTP2
aroA:CP4syn
LB
E9 3'

Figure 9.

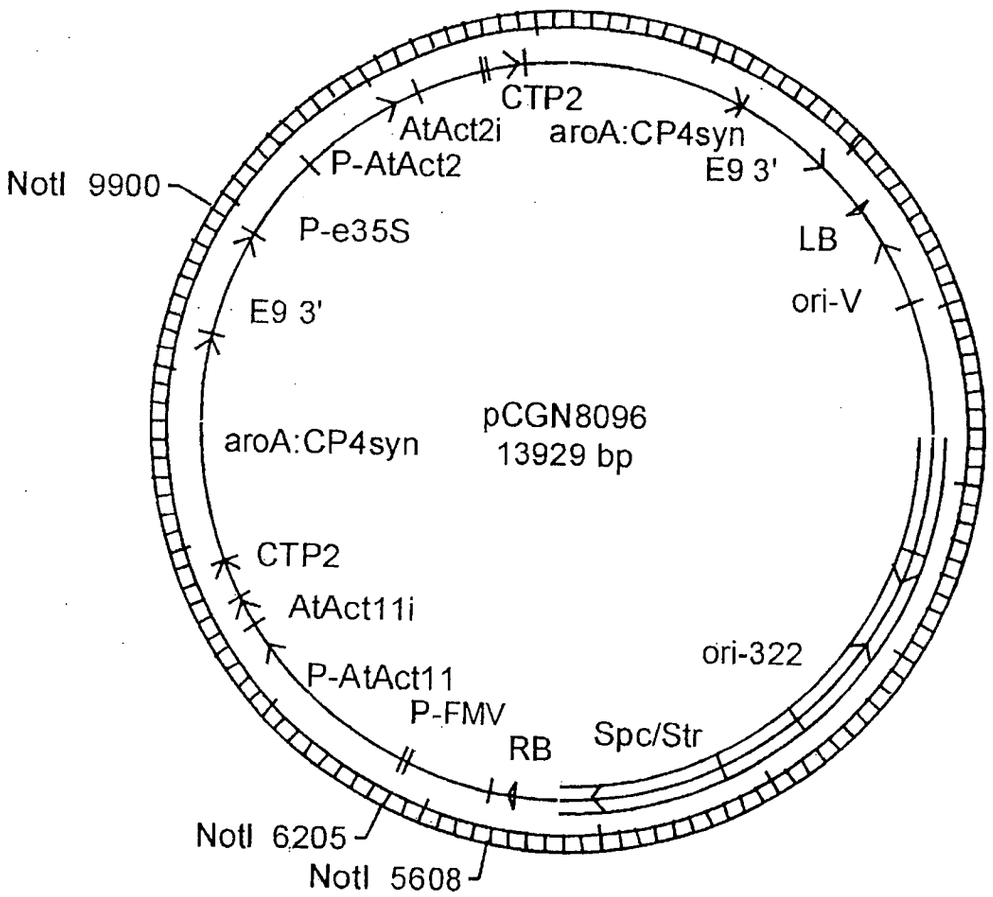


FIG. 10

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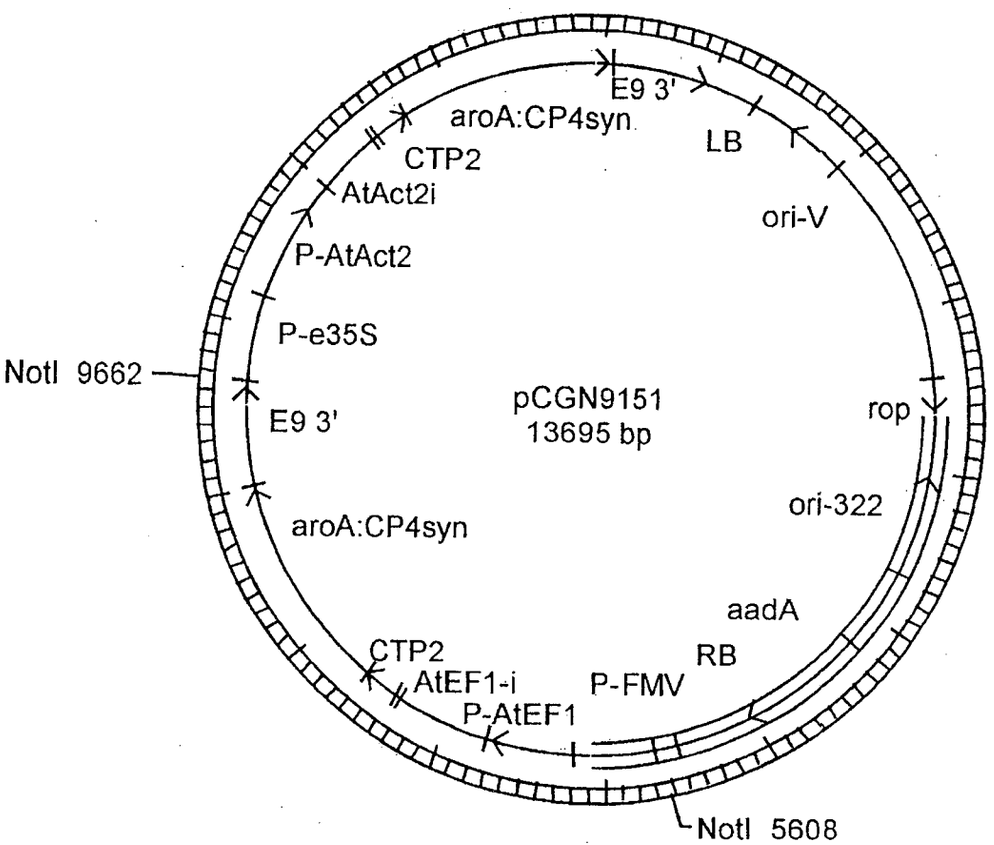

E9 3'
aroA:CP4syn
CTP2
AtAct2i
P-AtAct2
P-e35S
NotI 9662
E9 3'
aroA:CP4syn
CTP2
AtEF1-i
P-AtEF1
P-FMV
RB
aadA
ori-322
rop
ori-V
LB
pCGN9151
13695 bp
NotI 5608

FIG. 11

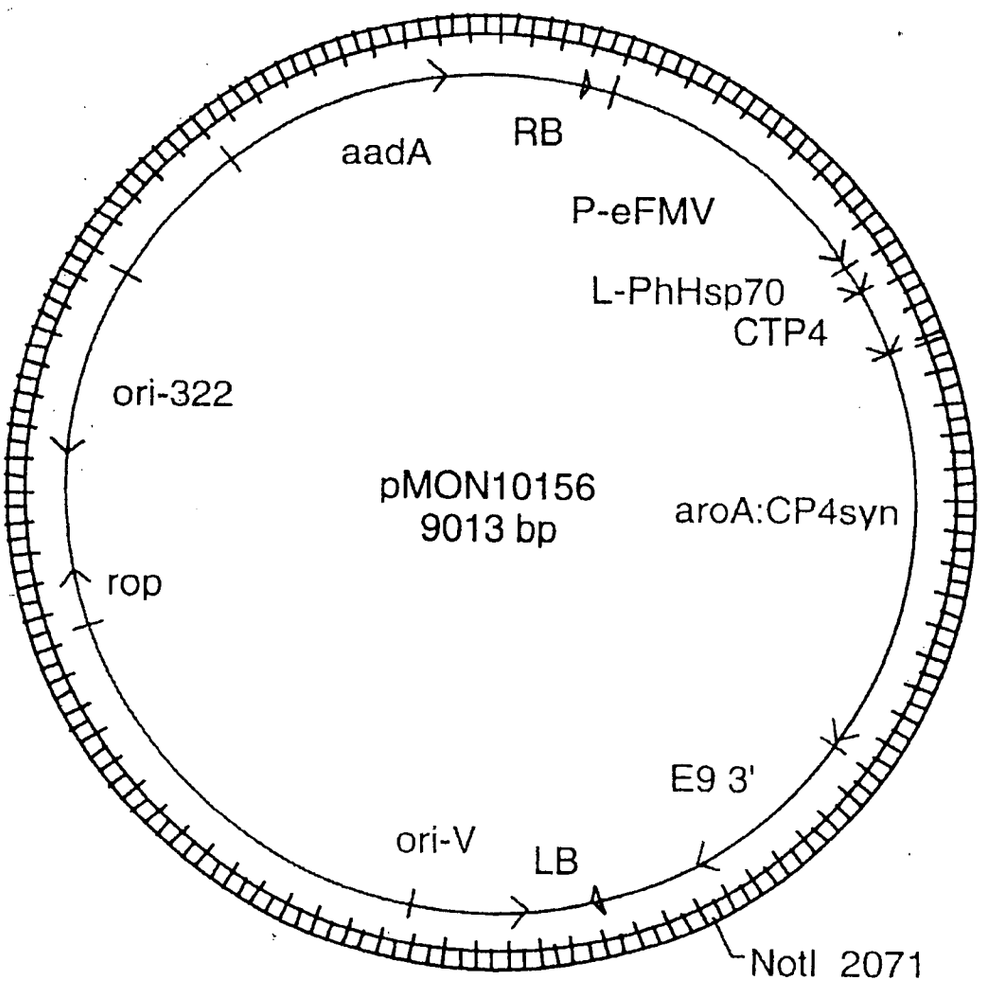


Figure 12

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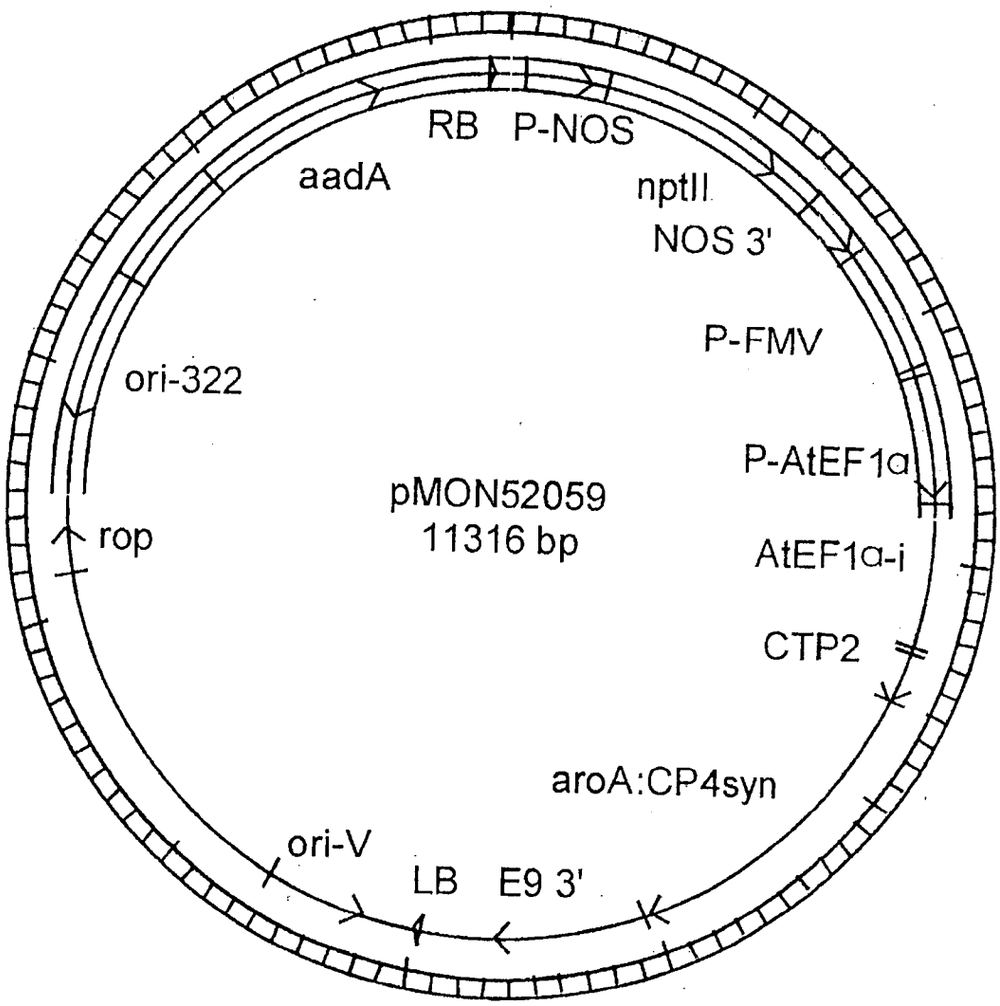

RB
P-NOS
aadA
nptII
NOS 3'
P-FMV
ori-322
P-AtEF1a
pMON52059
11316 bp
rop
AtEF1a-i
CTP2
aroA:CP4syn
ori-V
LB
E9 3'

FIG. 13

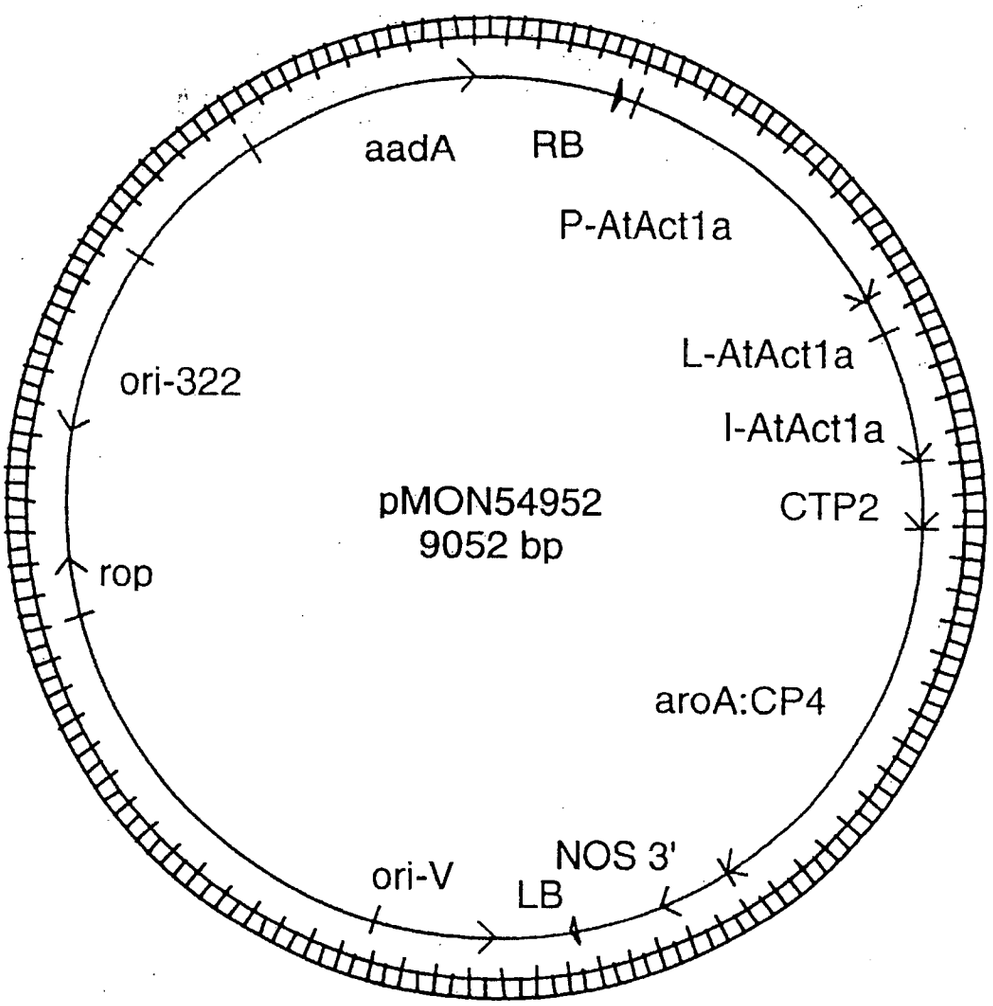


Figure 14

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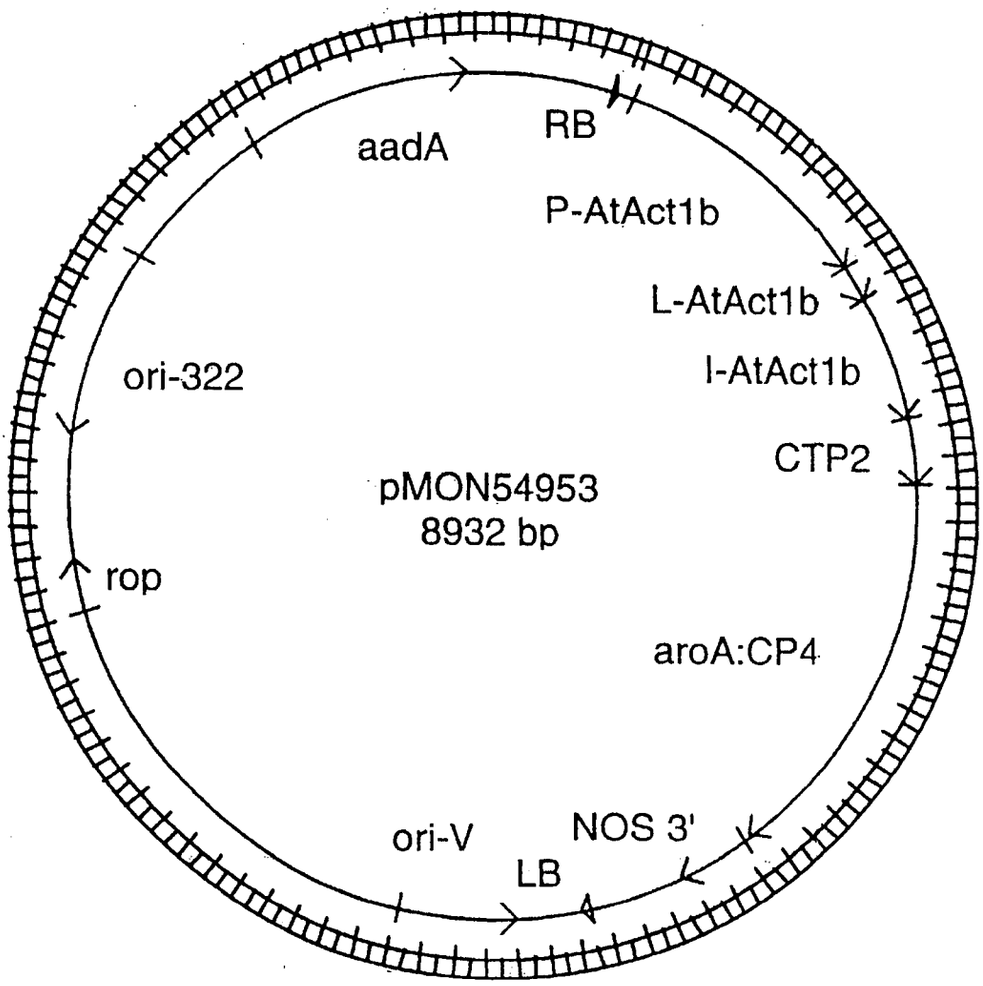

RB
aadA
P-AtAct1b
L-AtAct1b
I-AtAct1b
CTP2
ori-322
pMON54953
8932 bp
rop
aroA:CP4
ori-V
NOS 3'
LB

Figure 15

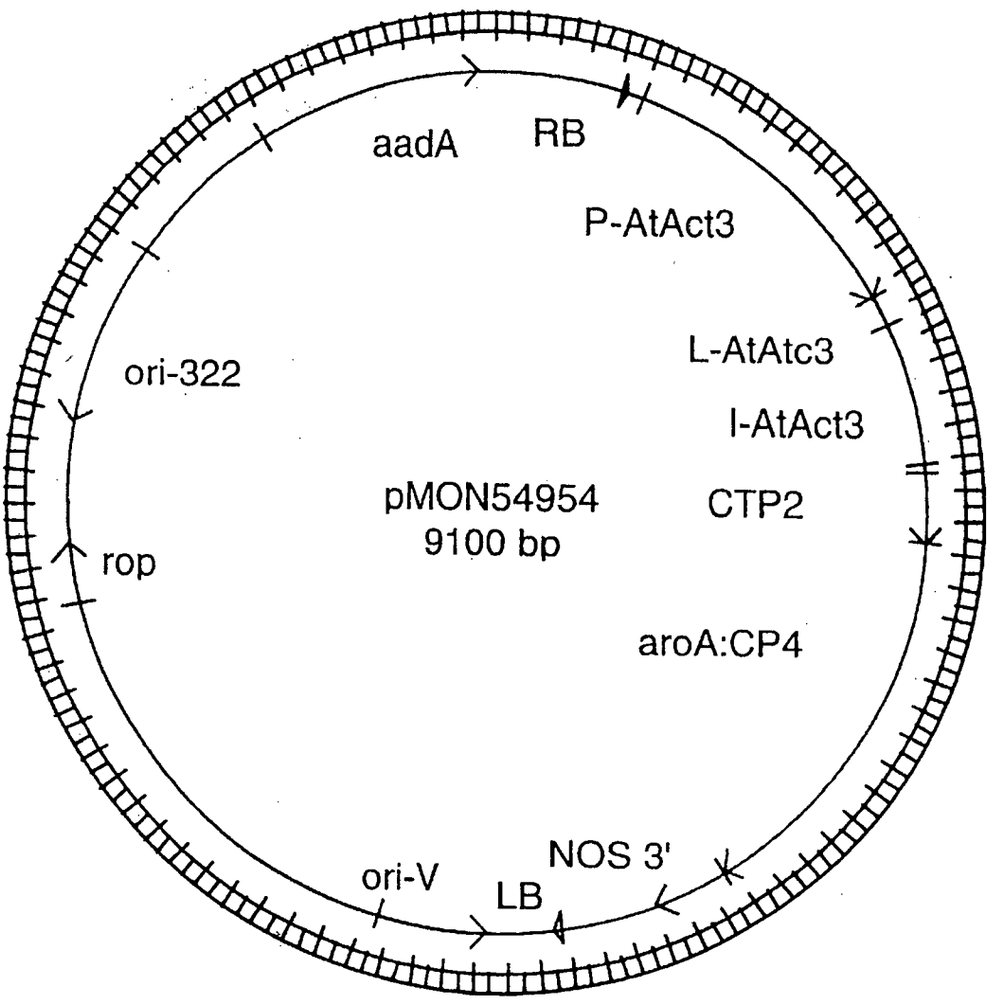


Figure 16

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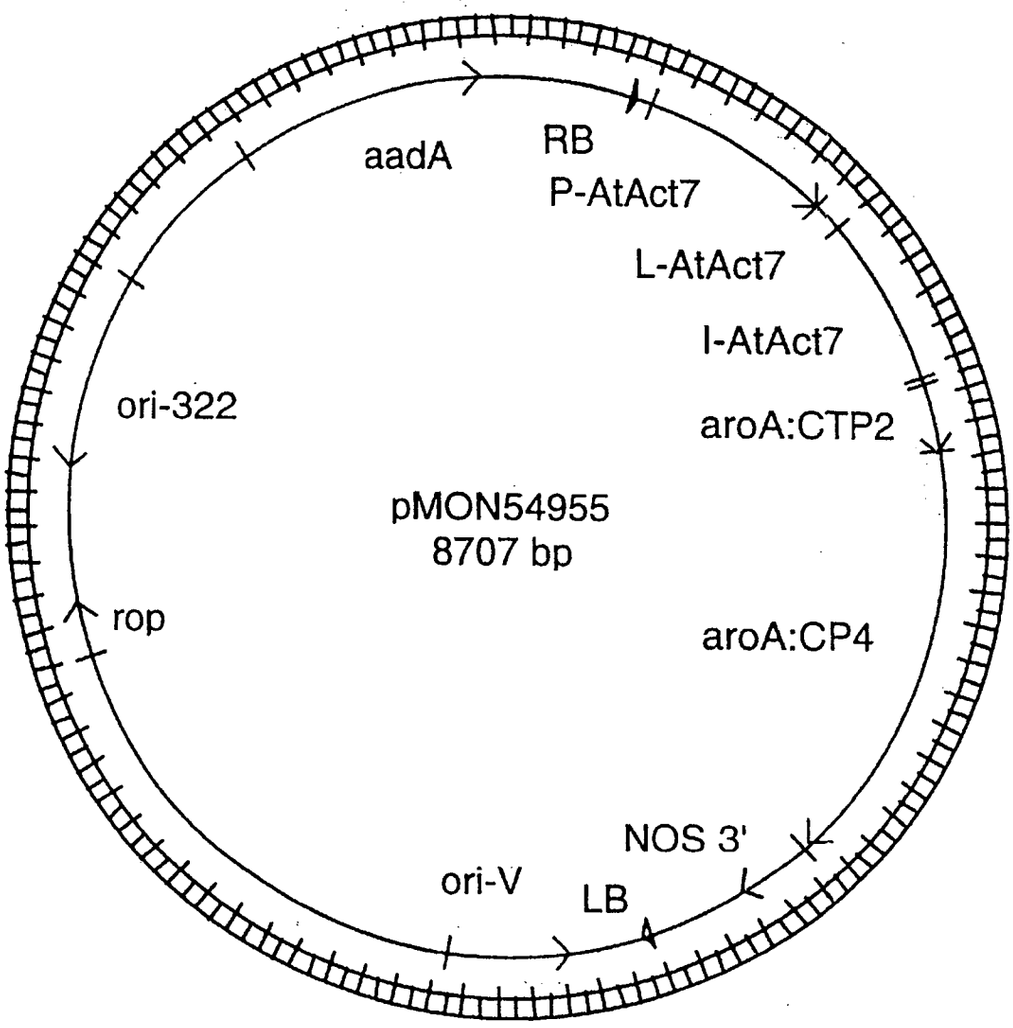

aadA
RB
P-AtAct7
L-AtAct7
I-AtAct7
aroA:CTP2
aroA:CP4
NOS 3'
LB
ori-V
rop
ori-322
pMON54955
8707 bp

Figure 17

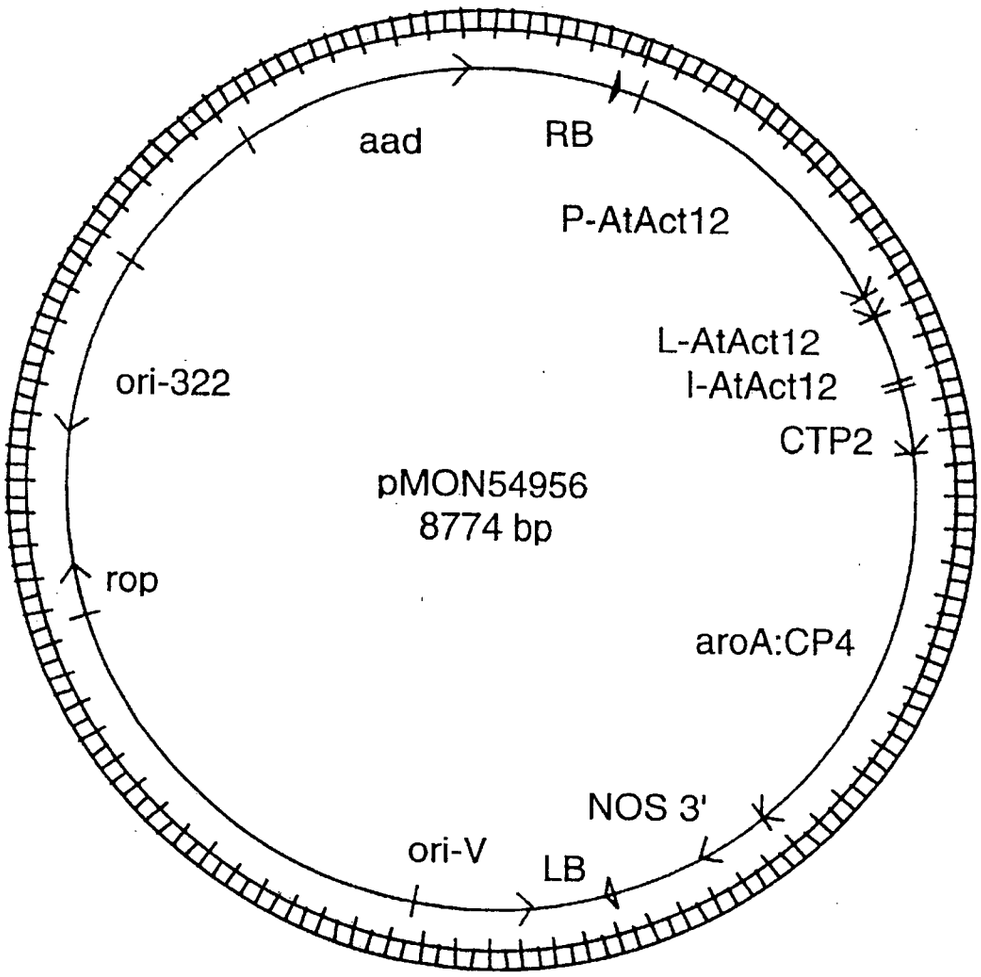


Figure 18

BNSDOCID: <US_____6462258B1_I_>

PLANT EXPRESSION CONSTRUCTS

CONTINUING DATA

This application claims priority to provisional application 60/171,173, filed Dec. 16, 1999.

FIELD OF THE INVENTION

The present invention relates to the isolation and use of nucleic acid molecules for control of gene expression in plants, specifically novel plant promoters and the combination of these promoters in an expression construct for plants.

BACKGROUND OF THE INVENTION

One of the goals of plant genetic engineering is to produce plants with agronomically important characteristics or traits. Recent advances in genetic engineering have provided the requisite tools to produce transgenic plants that contain and express foreign genes (Kahl et al., World J. of Microbiol. Biotech. 11:449–460, 1995). Particularly desirable traits or qualities of interest for plant genetic engineering would include but are not limited to resistance to insects, fungal diseases, and other pests and disease-causing agents, tolerances to herbicides, enhanced stability or shelf-life, yield, environmental tolerances, and nutritional enhancements. The technological advances in plant transformation and regeneration have enabled researchers to take exogenous DNA, such as a gene or genes from a heterologous or a native source, and incorporate the exogenous DNA into the plant's genome. In one approach, expression of a novel gene that is not normally expressed in a particular plant or plant tissue may confer a desired phenotypic effect. In another approach, transcription of a gene or part of a gene in an antisense orientation may produce a desirable effect by preventing or inhibiting expression of an endogenous gene.

In order to produce a transgenic plant, a construct that includes a heterologous gene sequence that confers a desired phenotype when expressed in the plant is introduced into a plant cell. The construct also includes a plant promoter that is operably linked to the heterologous gene sequence, often a promoter not normally associated with the heterologous gene. The construct is then introduced into a plant cell to produce a transformed plant cell, and the transformed plant cell is regenerated into a transgenic plant. The promoter controls expression of the introduced DNA sequence to which the promoter is operably linked and thus affects the desired characteristic conferred by the DNA sequence.

It would be advantageous to have a variety of promoters to tailor gene expression such that a gene or gene(s) is transcribed efficiently at the right time during plant growth and development, in the optimal location in the plant, and in the amount necessary to produce the desired effect. For example, constitutive expression of a gene product may be beneficial in one location of the plant but less beneficial in another part of the plant. In other cases, it may be beneficial to have a gene product produced at a certain developmental stage of the plant or in response to certain environmental or chemical stimuli. The commercial development of genetically improved germplasm has also advanced to the stage of introducing multiple traits into crop plants, often referred to as a gene stacking approach. In this approach, multiple genes conferring different characteristics of interest can be introduced into a plant. It is important when introducing multiple genes into a plant that each gene is modulated or controlled for optimal expression and that the regulatory elements are diverse in order to reduce the potential of gene

silencing. In light of these and other considerations, it is apparent that optimal control of gene expression and regulatory element diversity are important in plant biotechnology.

SUMMARY OF THE INVENTION

The present invention relates to DNA plant expression constructs that comprise Arabidopsis actin (Act) promoter sequences Act1a, Act1b, and the elongation factor 1α (EF1α) promoter sequence, and fragments and cis elements derived from these promoters operably linked to heterologous structural gene sequences that function in crop plant cells.

Thus, according to one embodiment of the invention, a recombinant DNA construct is provided that comprises, in operable linkage, a promoter that is functional in a cell of a crop plant, the promoter comprising: at least one cis element derived from SEQ ID NO:12, SEQ ID NO:22, and SEQ ID NO:23, a structural DNA sequence heterologous to the promoter; and a 3' non-translated region that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. For example, the promoter may consist essentially of a 5' regulatory region derived from any of SEQ ID NO:12, SEQ ID NO:22, and SEQ ID NO:23, (including or excluding any intron sequences located therein). The structural gene may comprise any heterologous nucleotide sequence wherein expression of the sequence results in an agronomically useful trait or product in a transgenic crop plant.

According to another aspect of the invention is a DNA construct comprising a structural DNA sequence operably linked to the promoter sequences of the present invention that encode a protein employed to confer herbicide tolerance to a crop plant. This herbicide tolerance protein includes, but is not limited to glyphosate tolerance protein genes such as a glyphosate resistant EPSP synthase gene alone, or in combination with one or more glyphosate degrading protein genes.

According to another embodiment of the invention, DNA constructs such as those described above are provided wherein the promoter is a hybrid or chimeric promoter comprising at least one cis element derived from one or more of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 operably linked to a heterologous promoter sequence such as a caulimovirus promoter, for example the Cauliflower mosaic virus 35S promoter or the Figwort mosaic virus promoter.

According to another embodiment of the invention, DNA constructs, such as those described above, are provided in tandem, wherein the promoter is a hybrid or chimeric promoter comprising at least one cis element derived from one or more of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 operably linked to a heterologous gene sequence that expresses in transgenic crop plant cells. The chimeric promoter sequences more specifically comprising the sequences identified in SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30.

According to another embodiment of the invention, a DNA construct such as that described above is provided wherein the structural DNA sequence is a glyphosate tolerance gene, such that when the DNA construct is introduced into a plant cell, it confers to the plant cell tolerance to an aqueous glyphosate formulation that includes at least 50

grams acid equivalent per liter (g a.e./l) of glyphosate. In other related embodiments, the DNA construct confers to the plant cell tolerance to glyphosate formulations having higher glyphosate concentrations (for example, at least 300 grams acid equivalent per liter of glyphosate. According to one embodiment, the DNA construct confers to the plant cell tolerance to at least one application of Roundup Ultra® at a rate of 16 ounces (oz) per acre, for example, and in other embodiments, glyphosate tolerance extends to one to two or more applications of 16 oz per acre, 32 oz per acre, or 64 oz per acre, for example.

According to another embodiment of the invention, transgenic crop plants are provided that are transformed with a DNA construct as described above, including monocot species and dicot species. We have discovered that the Arabidopsis actin and Arabidopsis EF1α promoters are sufficiently active in other crop plant species such as cotton, tomato, and sunflower, for example, that when used to control expression of a glyphosate tolerance gene, such as aroA:CP4, the plants tolerate commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

According to another embodiment of the invention, transgenic crop plants are provided that are transformed with multiple DNA constructs comprising the Arabidopsis actin and Arabidopsis EF1α promoters are sufficiently active in other plant species such as cotton, tomato, sunflower, for example, that when used to control expression of a glyphosate tolerance gene such as aroA:CP4, the plants tolerated commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

According to another embodiment of the invention, transgenic crop plants are provided that are transformed with DNA constructs comprising the Arabidopsis actin and Arabidopsis EF1α promoters as chimeric DNA molecules in fusion with caulimovirus DNA molecules having promoter activity in plants sufficiently active in other plant species such as cotton, tomato, canola, soybean, and sunflower, for example, that when used to control expression of a glyphosate tolerance gene such as aroA:CP4, the plants tolerate commercial application rates of glyphosate, exhibiting good vegetative tolerance and low damage to reproductive tissues. Such promoters can also be used to express other genes of interest in plants, including, but not limited to, genes that confer herbicide tolerance, insect control, disease resistance, increased stability or shelf, higher yield, nutritional enhancement, expression of a pharmaceutical or other desired polypeptide product, or a desirable change in plant physiology or morphology, and so on.

According to another embodiment of the invention methods are provided for expressing a structural DNA sequence in a plant. Such methods comprise, providing a DNA construct as described above, introducing the DNA construct into a plant cell, and regenerating the plant cell to produce a plant such that the structural DNA is expressible in the plant. According to a related embodiment, a method of controlling weeds is provided in which the DNA construct comprises a glyphosate tolerance gene and one applies to a crop plant transformed with the DNA construct an amount of glyphosate that is sufficient to control weeds without significantly damaging the crop plant.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plasmid map of pCGN8086.

FIG. **2** is a plasmid map of pMON45325

FIG. **3** is a plasmid map of pMON45331

FIG. **4** is a plasmid map of pMON45332

FIG. **5** is a plasmid map of pCGN9190

FIG. **6** is a plasmid map of pCGN9153

FIG. **7** is a plasmid map of pCGN8099

FIG. **8** is a plasmid map of pCGN8088

FIG. **9** is a plasmid map of pCGN8068

FIG. **10** is a plasmid map of pCGN8096

FIG. **11** is a plasmid map of pCGN9151

FIG. **12** is a plasmid map of pMON10156

FIG. **13** is a plasmid map of pMON52059

FIG. **14** is a plasmid map of pMON54952

FIG. **15** is a plasmid map of pMON54953

FIG. **16** is a plasmid map of pMON54954

FIG. **17** is a plasmid map of pMON54955

FIG. **18** is a plasmid map of pMON54956

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 is the forward PCR primer used for the isolation of the Act2 promoter.

SEQ ID NO:2 is the reverse PCR primer used for the isolation of the Act2 promoter.

SEQ ID NO:3 is the forward PCR primer used for the isolation of the Act8 promoter.

SEQ ID NO:4 is the reverse PCR primer used for the isolation of the Act8 promoter.

SEQ ID NO:5 is the forward PCR primer used for the isolation of the Act11 promoter.

SEQ ID NO:6 is the reverse PCR primer used for the isolation of the Act11 promoter.

SEQ ID NO:7 is the forward PCR primer used for the isolation of the EF1 promoter.

SEQ ID NO:8 is the reverse PCR primer used for the isolation of the EF1 promoter.

SEQ ID NO:9 is the sequence of the Act2 promoter including the intron sequence of the Act2 gene. Base positions 1–764 represent the promoter sequence; base positions 765–1215 represent the intron followed by 5 bases of 5' untranslated region (5' UTR) prior to the ATG; the transcription start site is located at base position 597.

SEQ ID NO:10 is the sequence of the Act8 promoter including the first intron of the Act8 gene. Base positions 1–797 represent the promoter sequence; base positions 798–1259 represent the intron followed by 10 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 646.

SEQ ID NO:11 is the sequence of the Act11 promoter including the first intron of the Act11 gene. Base positions 1–1218 represent the promoter sequence; base positions 1219–1381 represent the intron followed by 10 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 1062.

SEQ ID NO:12 is the sequence of the EF1 promoter including the first intron of the EF1 gene. Base positions 1–536 represent the promoter sequence; base positions 537–1137 represent the intron followed by 22 bases of 5' UTR prior to the ATG; the transcription start site is located at base position 481.

SEQ ID NO:13 is the forward PCR primer used for the isolation of the Act1a promoter.

SEQ ID NO:14 is the forward PCR primer used for the isolation of the Act1b promoter.

SEQ ID NO:15 is the reverse PCR primer used for the isolation of the Act1a and Act1b promoter.

SEQ ID NO:16 is the forward PCR primer used for the isolation of the Act3 promoter.

SEQ ID NO:17 is the reverse PCR primer used for the isolation of the Act3 promoter.

SEQ ID NO:18 is the forward PCR primer used for the isolation of the Act7 promoter.

SEQ ID NO:19 is the reverse PCR primer used for the isolation of the Act7 promoter.

SEQ ID NO:20 is the forward PCR primer used for the isolation of the Act12 promoter.

SEQ ID NO:21 is the reverse PCR primer used for the isolation of the Act12 promoter.

SEQ ID NO:22 is the sequence of the Act1a promoter including the first intron of the Act1a gene. Base positions 1–1033 represent the promoter sequence; base positions 1034–1578 represent the intron and 5' UTR.

SEQ ID NO:23 is the sequence of the Act1b promoter including the first intron of the Act1b gene. Base positions 1–914 represent the promoter sequence; base positions 915–1468 represent the intron and 5' UTR sequence.

SEQ ID NO:24 is the sequence of the Act3 promoter including the first intron of the Act3 gene. Base positions 1–1023 represent the promoter sequence; base positions 1024–1642 represent the intron and 5' UTR sequence.

SEQ ID NO:25 is the sequence of the Act7 promoter including the first intron of the Act7 gene. Base positions 1–600 represent the promoter sequence; base positions 601–1241 represent the intron and 5' UTR sequence.

SEQ ID NO:26 is the sequence of the Act12 promoter including the first intron of the Act12 gene. Base positions 1–1017 represent the promoter sequence; base positions 1018–1313 represent the intron and 5' UTR sequence.

SEQ ID NO:27 is the sequence of the chimeric FMV-Act11 promoter including the first intron of the Act11 gene. Base positions 1–536 represent the FMV promoter sequence; base positions 553–1946 represent the Arabidopsis Actin 11 promoter, intron and 5' UTR sequence.

SEQ ID NO:28 is the sequence of the chimeric FMV-EF1α promoter including the first intron of the EF1α gene. Base positions 1–536 represent the FMV promoter sequence; base positions 553–1695 represent the EF1α promoter, intron and 5' UTR sequence.

SEQ ID NO:29 is the sequence of the CaMV-Act8 promoter including the first intron of the Act8 gene. Base positions 1–523 present the CaMV promoter sequence; base positions 534–1800 represent the Act8 promoter, intron and 5' UTR sequence.

SEQ ID NO:30 is the sequence of the CaMV-Act2 promoter including the first intron of the Act2 gene. Base positions 1–523 represent the CaMV promoter sequence; base positions 534–1742 represent the Act2 promoter, intron and 5' UTR sequence.

DETAILED DESCRIPTION OF THE INVENTION

This application claims the benefit of U.S. Provisional Application No. 60/171,173, filed Dec. 16, 1999. The following definitions and methods are provided to better define, and to guide those of ordinary skill in the art in the practice of, the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used. The standard one- and three-letter nomenclature for amino acid residues is used.

"Nucleic acid (sequence)" or "polynucleotide (sequence)" refers to single- or double-stranded DNA or RNA of genomic or synthetic origin, i.e., a polymer of deoxyribonucleotide or ribonucleotide bases, respectively, read from the 5' (upstream) end to the 3' (downstream) end. The nucleic acid can represent the sense or complementary (antisense) strand.

"Native" refers to a naturally occurring ("wild-type") nucleic acid sequence.

"Heterologous" sequence refers to a sequence which originates from a foreign source or species or, if from the same source, is modified from its original form.

An "isolated" nucleic acid sequence is substantially separated or purified away from other nucleic acid sequences with which the nucleic acid is normally associated in the cell of the organism in which the nucleic acid naturally occurs, i.e., other chromosomal or extrachromosomal DNA. The term embraces nucleic acids that are biochemically purified so as to substantially remove contaminating nucleic acids and other cellular components. The term also embraces recombinant nucleic acids and chemically synthesized nucleic acids.

The term "substantially purified", as used herein, refers to a molecule separated from other molecules normally associated with it in its native state. More preferably, a substantially purified molecule is the predominant species present in a preparation. A substantially purified molecule may be greater than 60% free, preferably 75% free, more preferably 90% free from the other molecules (exclusive of solvent) present in the natural mixture. The term "substantially purified" is not intended to encompass molecules present in their native state.

A first nucleic acid sequence displays "substantially identity" to a reference nucleic acid sequence if, when optimally aligned (with appropriate nucleotide insertions or deletions totaling less than 20 percent of the reference sequence over the window of comparison) with the other nucleic acid (or its complementary strand), there is at least about 75% nucleotide sequence identity, preferably at least about 80% identity, more preferably at least about 85% identity, and most preferably at least about 90% identity over a comparison window of at least 20 nucleotide positions, preferably at least 50 nucleotide positions, more preferably at least 100 nucleotide positions, and most preferably over the entire length of the first nucleic acid. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482, 1981; by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443, 1970; by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; preferably by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA) in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis. The reference nucleic acid may be a full-length molecule or a portion of a longer molecule. Alternatively, two nucleic acids are have substantial identity if one hybridizes to the other under stringent conditions, as defined below.

A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the sequences are so arranged that the first nucleic acid sequence affects the function of the second nucleic-acid sequence. Preferably, the two sequences are part of a single contiguous nucleic acid molecule and more preferably are adjacent. For example, a promoter is operably linked to a gene if the promoter regulates or mediates transcription of the gene in a cell.

A "recombinant" nucleic acid is made by an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. Techniques for nucleic-acid manipulation are well-known (see for example Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press, 1995; Birren et al., Genome Analysis: volume 1, Analyzing DNA, (1997), volume 2, Detecting Genes, (1998), volume 3, Cloning Systems, (1999) volume 4, Mapping Genomes, (1999), Cold Spring Harbor, N.Y.).

Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859–1862, 1981, and Matteucci et al., J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers.

A "synthetic nucleic acid sequence" can be designed and chemically synthesized for enhanced expression in particular host cells and for the purposes of cloning into appropriate constructs. Host cells often display a preferred pattern of codon usage (Murray et al., 1989). Synthetic DNAs designed to enhance expression in a particular host should therefore reflect the pattern of codon usage in the host cell. Computer programs are available for these purposes including but not limited to the "BestFit" or "Gap" programs of the Sequence Analysis Software Package, Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, Wis. 53711.

"Amplification" of nucleic acids or "nucleic acid reproduction" refers to the production of additional copies of a nucleic acid sequence and is carried out using polymerase chain reaction (PCR) technologies. A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in PCR Protocols: A Guide to Methods and Applications, ed. Innis et al., Academic Press, San Diego, 1990. In PCR, a primer refers to a short oligonucleotide of defined sequence which is annealed to a DNA template to initiate the polymerase chain reaction.

"Transformed", "transfected", or "transgenic" refers to a cell, tissue, organ, or organism into which has been introduced a foreign nucleic acid, such as a recombinant construct. Preferably, the introduced nucleic acid is integrated into the genomic DNA of the recipient cell, tissue, organ or organism such that the introduced nucleic acid is inherited by subsequent progeny. A "transgenic" or "transformed" cell or organism also includes progeny of the cell or organism and progeny produced from a breeding program employing such a "transgenic" plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a recombinant construct or construct.

The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression. Some genes can be transcribed into mRNA and translated into polypeptides (structural genes); other genes can be transcribed into RNA (e.g. rRNA, tRNA); and other types of gene function as regulators of expression (regulator genes).

"Expression" of a gene refers to the transcription of a gene to produce the corresponding mRNA and translation of this mRNA to produce the corresponding gene product, i.e., a peptide, polypeptide, or protein. Gene expression is controlled or modulated by regulatory elements including 5' regulatory elements such as promoters.

"Genetic component" refers to any nucleic acid sequence or genetic element which may also be a component or part of an expression construct. Examples of genetic components include, but are not limited to promoter regions, 5' untranslated leaders, introns, genes, 3' untranslated regions, and other regulatory sequences or sequences which affect transcription or translation of one or more nucleic acid sequences.

The terms "recombinant DNA construct", "recombinant construct", "expression construct" or "expression cassette" refer to any agent such as a plasmid, cosmid, virus, BAC (bacterial artificial chromosome), autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a DNA molecule in which one or more DNA sequences have been linked in a functionally operative manner using well-known recombinant DNA techniques.

"Complementary" refers to the natural association of nucleic acid sequences by base-pairing (A-G-T pairs with the complementary sequence T-C-A). Complementarity between two single-stranded molecules may be partial, if only some of the nucleic acids pair are complementary; or complete, if all bases pair are complementary. The degree of complementarity affects the efficiency and strength of hybridization and amplification reactions.

"Homology" refers to the level of similarity between nucleic acid or amino acid sequences in terms of percent nucleotide or amino acid positional identity, respectively, i.e., sequence similarity or identity. Homology also refers to the concept of similar functional properties among different nucleic acids or proteins.

"Promoter" refers to a nucleic acid sequence located upstream or 5' to a translational start codon of an open reading frame (or protein-coding region) of a gene and that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) to initiate transcription. A "plant promoter" is a native or non-native promoter that is functional in plant cells. Constitutive promoters are functional in most or all tissues of a plant throughout plant development. Tissue-, organ- or cell-specific promoters are expressed only or predominantly in a particular tissue, organ, or cell type, respectively. Rather than being expressed "specifically" in a given tissue, organ, or cell type, a promoter may display "enhanced" expression, i.e., a higher level of expression, in one part (e.g., cell type, tissue, or organ) of the plant compared to other parts of the plant. Temporally regulated promoters are functional only or predominantly during certain periods of plant development or at certain times of day, as in the case of genes associated with circadian rhythm, for example. Inducible promoters selectively express an operably linked DNA sequence in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical

inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters regulated by light, heat, stress, flooding or drought, phytohormones, wounding, or chemicals such as ethanol, jasmonate, salicylic acid, or safeners.

Any plant promoter can be used as a 5' regulatory sequence for modulating expression of a particular gene or genes. One preferred promoter would be a plant RNA polymerase II promoter. Plant RNA polymerase II promoters, like those of other higher eukaryotes, have complex structures and are comprised of several distinct elements. One such element is the TATA box or Goldberg-Hogness box, which is required for correct expression of eukaryotic genes in vitro and accurate, efficient initiation of transcription in vivo. The TATA box is typically positioned at approximately −25 to −35, that is, at 25 to 35 basepairs (bp) upstream (5') of the transcription initiation site, or cap site, which is defined as position +1 (Breathnach and Chambon, Ann. Rev. Biochem. 50:349–383, 1981; Messing et al., In: Genetic Engineering of Plants, Kosuge et al., eds., pp. 211–227, 1983). Another common element, the CCAAT box, is located between −70 and −100 bp. In plants, the CCAAT box may have a different consensus sequence than the functionally analogous sequence of mammalian promoters (the plant analogue has been termed the "AGGA box" to differentiate it from its animal counterpart; Messing et al., In: Genetic Engineering of Plants, Kosuge et al., eds., pp. 211–227, 1983). In addition, virtually all promoters include additional upstream activating sequences or enhancers (Benoist and Chambon, Nature 290:304–310, 1981; Gruss et al., Proc. Nat. Acad. Sci. USA 78:943–947, 1981; and Khoury and Gruss, Cell 27:313–314, 1983) extending from around −100 bp to −1,000 bp or more upstream of the transcription initiation site.

When fused to heterologous DNA sequences, such promoters typically cause the fused sequence to be transcribed in a manner that is similar to that of the gene sequence with which the promoter is normally associated. Promoter fragments that include regulatory sequences can be added (for example, fused to the 5' end of, or inserted within, an active promoter having its own partial or complete regulatory sequences (Fluhr et al., Science 232:1106–1112, 1986; Ellis et al., EMBO J. 6:11–16, 1987; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986–8990, 1987; Poulsen and Chua, Mol. Gen. Genet. 214:16–23, 1988; Comai et al., Plant Mol. Biol. 15:373–381, 1991). Alternatively, heterologous regulatory sequences can be added to the 5' upstream region of an inactive, truncated promoter, e.g., a promoter including only the core TATA and, sometimes, the CCAAT elements (Fluhr et al., Science 232:1106–1112, 1986; Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986–8990, 1987; Aryan et al., Mol. Gen. Genet. 225:65–71, 1991).

Promoters are typically comprised of multiple distinct "cis-acting transcriptional regulatory elements," or simply "cis-elements," each of which confers a different aspect of the overall control of gene expression (Strittmatter and Chua, Proc. Nat. Acad. Sci. USA 84:8986–8990, 1987; Ellis et al., EMBO J. 6:11–16, 1987; Benfey et al., EMBO J. 9:1677–1684, 1990). "Cis elements" bind trans-acting protein factors that regulate transcription. Some cis elements bind more than one factor, and trans-acting transcription factors may interact with different affinities with more than one cis element (Johnson and McKnight, Ann. Rev. Biochem. 58:799–839, 1989). Plant transcription factors, corresponding cis elements, and analysis of their interaction are discussed, for example, In: Martin, Curr. Opinions Biotech. 7:130–138, 1996; Murai, In: Methods in Plant Biochemistry and Molecular Biology, Dashek, ed., CRC Press, 1997, pp. 397–422; and Methods in Plant Molecular Biology, Maliga et al., eds., Cold Spring Harbor Press, 1995, pp. 233–300. The promoter sequences of the present invention can contain "cis elements" that confer or modulate gene expression.

Cis elements can be identified by a number of techniques, including deletion analysis, i.e., deleting one or more nucleotides from the 5' end or internal to a promoter; DNA binding protein analysis using Dnase I footprinting, methylation interference, electrophoresis mobility-shift assays, in vivo genomic footprinting by ligation-mediated PCR and other conventional assays; or by sequence similarity with known cis element motifs by conventional sequence comparison methods. The fine structure of a cis element can be further studies by mutagenesis (or substitution) of one or more nucleotides of the element or by other conventional methods (see for example, Methods in Plant Biochemistry and Molecular Biology, Dashek, ed., CRC Press, 1997, pp. 397–422; and Methods in Plant Molecular Biology, Maliga et al., eds., Cold Spring Harbor Press, 1995, pp. 233–300).

Cis elements can be obtained by chemical synthesis or by cloning from promoters that include such elements. Cis elements can also be synthesized with additional flanking sequences that contain useful restriction enzyme sites to facilitate subsequence manipulation. In one embodiment, the promoters are comprised of multiple distinct "cis-elements". In a preferred embodiment sequence regions comprising "cis elements" of the nucleic acid sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 are identified using computer programs including, but not limited to MEME or SIGNALSCAN that are designed specifically to identify cis elements, or domains or motifs within sequences.

The present invention includes fragments or cis elements of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 or homologues of cis elements known to effect gene regulation that show homology with the nucleic acid sequences of the present invention. Such nucleic acid fragments can include any region of the disclosed sequences. The promoter regions or partial promoter regions of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 can contain at least one regulatory element including, but not limited to "cis elements" or domains that are capable of regulating expression of operably linked DNA sequences, such as in male reproductive tissues.

Plant promoters can also include promoters produced through the manipulation of known promoters to produce synthetic, chimeric, or hybrid promoters. Such promoters can also combine cis elements from one or more promoters, for example, by adding a heterologous regulatory sequence to an active promoter with its own partial or complete regulatory sequences (Ellis et al., EMBO J. 6:11–16, 1987; Strittmatter and Chua, Proc. Nat Acad. Sci. USA 84:8986–8990, 1987; Poulsen and Chua, Mol. Gen. Genet. 214:16–23, 1988; Comai et al., Plant. Mol. Biol. 15:373–381, 1991). Chimeric promoters have also been developed by adding a heterologous regulatory sequence to the 5' upstream region of an inactive, truncated promoter, i.e., a promoter that includes only the core TATA and, optionally, the CCAAT elements (Fluhr et al., Science 232:1106–1112, 1986; Strittmatter and Chua, Proc. Nat.

Acad. Sci. USA 84:8986–8990, 1987; Aryan et al., Mol. Gen. Genet. 225:65–71, 1991).

Chimeric or hybrid promoters according to the present invention may include at least one known cis element such as elements that are regulated by numerous environmental factors such as light, heat, or stress; elements that are regulated or induced by pathogens or chemicals, and the like. Such elements may either positively or negatively regulate gene expression, depending on the conditions. Examples of cis elements include, but are not limited to oxygen responsive elements (Cowen et al., J. Biol. Chem. 268(36):26904, 1993), light regulatory elements (see for example, Bruce and Quail, Plant Cell 2: 1081, 1990, and Bruce et al., EMBO J. 10:3015, 1991, a cis element reponsive to methyl jasmonate treatment (Beaudoin and Rothstein, Plant Mol. Biol. 33:835, 1997, salicylic acid-responsive elements (Strange et al., Plant J. 11:1315, 1997, heat shock response elements (Pelham et al., Trends Genet. 1:31, 1985, elements responsive to wounding and abiotic stress (Loace et al., Proc. Natl. Acad. Sci. U.S.A. 89:9230, 1992; Mhiri et al., Plant Mol. Biol. 33:257, 1997), cold-responsive elements (Baker et al., Plant Mol. Biol. 24:701, 1994; Jiang et al., Plant Mol. Biol. 30:679, 1996; Nordin et al., Plant Mol. Biol. 21:641, 1993; Zhou et al., J. Biol. Chem. 267:23515, 1992), and drought responsive elements, (Yamaguchi et al., Plant Cell 6:251–264, 1994; Wang et al., Plant Mol. Biol. 28:605, 1995; Bray E. A. Trends in Plant Science 2:48, 1997).

In another embodiment, the nucleotide sequences as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 includes any length of said nucleotide sequences that is capable of regulating an operably linked DNA sequence. For example, the sequences as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 may be truncated or have portions deleted and still be capable of regulating transcription of an operably linked DNA sequence. In a related embodiment, a cis element of the disclosed sequences may confer a particular specificity such as conferring enhanced expression of operably linked DNA sequences in certain tissues. Consequently, any sequence fragments, portions, or regions of the disclosed sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 can be used as regulatory sequences including but not limited to cis elements or motifs of the disclosed sequences. For example, one or more base pairs may be deleted from the 5' or 3' end of a promoter sequence to produce a "truncated" promoter. One or more base pairs can also be inserted, deleted, or substituted internally to a promoter sequence. Promoters can be constructed such that promoter fragments or elements are operably linked, for example, by placing such a fragment upstream of a minimal promoter. A minimal or basal promoter is a piece of DNA which is capable of recruiting and binding the basal transcription machinery. One example of basal transcription machinery in eukaryotic cells is the RNA polymerase II complex and its accessory proteins. The enzymatic components of the basal transcription machinery are capable of initiating and elongating transcription of a given gene, utilizing a minimal or basal promoter. That is, there are not added cis-acting sequences in the promoter region that are capable of recruiting and binding transcription factors that modulate transcription, e.g., enhance, repress, render transcription hormone-dependent, etc. Substitutions, deletions, insertions or any combination thereof can be combined to produce a final construct.

The promoter sequences of the present invention may be modified, for example for expression in other plant systems. In another approach, novel hybrid promoters can be designed or engineered by a number of methods. Many promoters contain upstream sequences which activate, enhance or define the strength and/or specificity of the promoter (Atchison, Ann. Rev. Cell Biol. 4:127, 1988). T-DNA genes; for example contain "TATA" boxes defining the site of transcription initiation and other upstream elements located upstream of the transcription initiation site modulate transcription levels (Gelvin, In: Transgenic Plants (Kung, S.-D. and Us,R., eds, San Diego: Academic Press, pp.49–87, 1988). Another chimeric promoter combined a trimer of the octopine synthase (ocs) activator to the mannopine synthase (mas) activator plus promoter and reported an increase in expression of a reporter gene (Min Ni et al., The Plant Journal 7:661, 1995). The upstream regulatory sequences of the present invention can be used for the construction of such chimeric or hybrid promoters. Methods for construction of variant promoters of the present invention include but are not limited to combining control elements of different promoters or duplicating portions or regions of a promoter (see for example U.S. Pat. Nos. 5,110,732 and 5,097,025). Those of skill in the art are familiar with the specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolation of genes, (see for example Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press, 1995; Birren et al., Genome Analysis: volume 1, Analyzing DNA, (1997), volume 2, Detecting Genes, (1998), volume 3, Cloning Systems, (1999) volume 4, Mapping Genomes, (1999), Cold Spring Harbor, N.Y.).

The design, construction, and use of chimeric or hybrid promoters comprising one or more of cis elements of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, for modulating or regulating the expression of operably linked nucleic acid sequences are also encompassed by the present invention.

The promoter sequences, fragments, regions or cis elements thereof of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 are capable of transcribing operably linked DNA sequences in multiple tissues and therefore can selectively regulate expression of genes in multiple tissues.

For a number of agronomic traits, transcription of a gene or genes of interest is desirable in multiple tissues to confer the desired characteristic(s). The availability of suitable promoters that regulate transcription of operably linked genes in selected target tissues of interest is desirable, since it may not be desirable to express a gene in every tissue, but only in certain tissues. For example, if one desires to selectively express a target gene for expression of gene for herbicide tolerance, one may desire expression of the herbicide tolerance gene in vegetative and reproductive tissues. The promoter sequences of the present invention are useful

for regulating gene expression in multiple tissues including, but not limited to rapidly growing meristematic tissues, male reproductive tissues (androecium) such as pollen, anthers, and filaments, and female reproductive tissues (gynoecium) such as the stigma, style, and ovaries, leaves, sepals, and petals. The promoters of the present invention therefore have utility for expression of herbicide tolerance genes, for example, where tolerance is desired in multiple tissues and stages of plant development. The promoter sequences of the present invention have utility for regulating transcription of any target gene including but not limited to genes for control of fertility, yield, insect tolerance, fungal tolerance, herbicide tolerance, or any desirable trait of interest. Particularly preferred genes include herbicide tolerance genes or insect tolerance genes.

In one embodiment, the promoters of the present invention have particular utility for regulating expression of an herbicide tolerance gene where expression of a gene is desired in multiple tissues. For example, the herbicide tolerance gene may confer tolerance to the herbicide glyphosate. Examples of suitable glyphosate tolerance genes include, but are not limited to glyphosate resistant EPSP synthase genes or gene products that degrade glyphosate such as, a glyphosate oxidoreductase and phosphonate N-acetyl transferase. It is important to have a wide variety of choices of 5' regulatory elements for any plant biotechnology strategy in order to have suitable regulatory elements that are most efficient for the expression profile desired.

In another embodiment, the promoters of the present invention have utility for determining gene function. The function of many genes is unknown and the promoters of the present invention can be used as genetic elements in a construct to allow a phenotypic evaluation of one or more genes expressed in a sense or antisense orientation. The promoters of the present invention can be components in a plant expression construct developed for a high throughput assay where high levels of gene expression in constitutive and reproductive tissues is desired.

Any plant can be selected for the identification of genes and regulatory sequences. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, Arabidopsis, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radisicchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini. Particularly preferred plants for the identification of regulatory sequences are Arabidopsis, corn, wheat, soybean, and cotton.

The promoter sequences of the present invention were isolated from *Arabidopsis thaliana* plant DNA. In a preferred embodiment, a construct includes the promoter sequences of the present invention operably linked to a transcribable sequence along with suitable terminator and regulatory elements. Such a construct may be transformed into a suitable target plant of interest. Any plant can be used as a suitable host for nucleic acid constructs comprising the promoter sequences of the present invention. Examples of suitable target plants of interest would include, but are not limited to alfalfa, broccoli, cabbage, canola, cauliflower, corn, cotton, cranberry, cucumber, lettuce, pea, poplar, pine, potato, onion, rice, raspberry, soybean, sugarcane, sugarbeet, sunflower, tomato, and wheat.

Promoter Isolation and Modification Methods

Any number of methods can be used to isolate fragments of the promoter sequences disclosed herein. A PCR-based approach can be used to amplify flanking regions from a genomic library of a plant using publicly available sequence information. A number of methods are known to those of skill in the art to amplify unknown DNA sequences adjacent to a core region of known sequence. Methods include but are not limited to inverse PCR (IPCR), vectorette PCR, Y-shaped PCR and genome walking approaches. For the present invention, the nucleic acid molecules were isolated from Arabidopsis by designing PCR primers based on available sequence information.

Nucleic acid fragments can also be obtained by other techniques such as by directly synthesizing the fragment by chemical means, as is commonly practiced by using an automated oligonucleotide synthesizer. Fragments can also be obtained by application of nucleic acid reproduction technology, such as the PCR (polymerase chain reaction) technology by recombinant DNA techniques generally known to those of skill in the art of molecular biology. Regarding the amplification of a target nucleic-acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent PCR conditions" refer to conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product.

Those of skill in the art are aware of methods for the preparation of plant genomic DNA. In one approach, genomic DNA libraries can be prepared from a chosen species by partial digestion with a restriction enzyme and size selecting the DNA fragments within a particular size range. The genomic DNA can be cloned into a suitable construct including but not limited to a bacteriophage, and prepared using a suitable construct such as a bacteriophage using a suitable cloning kit from any number of vendors (see for example Stratagene, La Jolla Calif. or Gibco BRL, Gaithersburg, Md.).

In another embodiment, the nucleotide sequences of the promoters disclosed herein can be modified. Those skilled in the art can create DNA molecules that have variations in the nucleotide sequence. The nucleotide sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 may be modified or altered to enhance their control characteristics. For example, the sequences may be modified by insertion, deletion or replacement of template sequences in a PCR-based DNA modification approach. "Variant" DNA molecules are DNA molecules containing changes in which one or more nucleotides of a native sequence is deleted, added, and/or substituted, preferably while substantially maintaining promoter function. In the case of a promoter fragment, "variant" DNA can include

changes affecting the transcription of a minimal promoter to which it is operably linked. Variant DNA molecules can be produced, for example, by standard DNA mutagenesis techniques or by chemically synthesizing the variant DNA molecule or a portion thereof.

In addition to their use in modulating gene expression, the promoter sequences of the present invention also have utility as probes or primers in nucleic acid hybridization experiments. The nucleic-acid probes and primers of the present invention can hybridize under stringent conditions to a target DNA sequence. The term "stringent hybridization conditions" is defined as conditions under which a probe or primer hybridizes specifically with a-target sequence(s) and not with non-target sequences, as can be determined empirically. The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by the specific hybridization procedure (see for example Sambrook et al., 1989, at 9.52–9.55, Sambrook et al., 1989 at 9.47–9.52, 9.56–9.58; Kanehisa, Nucl. Acids Res. 12:203–213, 1984; and Wetmur and Davidson, J. Mol. Biol. 31:349–370, 1968). Appropriate stringency conditions which promote DNA hybridization are, for example, 6.0× sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2.0×SSC at 50° C., are known to those skilled in the art or can be found in laboratory manuals including but not limited to Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 1989, 6.3.1–6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0×SSC at 50° C. to a high stringency of about 0.2×SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C., to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. For example, hybridization using DNA or RNA probes or primers can be performed at 65° C. in 6×SSC, 0.5% SDS, 5×Denhardt's, 100 μg/mL nonspecific DNA (e.g., sonicated salmon sperm DNA) with washing at 0.5× SSC, 0.5% SDS at 65° C., for high stringency.

A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low stringency" conditions. Similarly, the molecules are said to be "complementary" is they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high stringency" conditions. It is contemplated that lower stringency hybridization conditions such as lower hybridization and/or washing temperatures can be used to identify related sequences having a lower degree of sequence similarity if specificity of binding of the probe or primer to target sequence(s) is preserved. Accordingly, the nucleotide sequences of the present invention can be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Detection of DNA segments via hybridization is well-known to those of skill in the art, and thus depending on the application envisioned, one will desire to employ varying hybridization conditions to achieve varying degrees of selectivity of probe towards target sequence and the method of choice will depend on the desired results. Conventional stringency conditions are described in Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989, and by Haymes et al., Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C., 1985.

In one embodiment of the present invention, the nucleic acid sequences SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26, or a fragment, region, cis element, or oligomer of these sequences, are used in hybridization assays of other plant tissues to identify closely related or homologous genes and associated regulatory sequences. These include but are not limited to Southern or northern hybridization assays on any substrate including but not limited to an appropriately prepared plant tissue, cellulose, nylon, or combination filter, chip, or glass slide. Such methodologies are well known in the art and are available in a kit or preparation which can be supplied by commercial vendors.

A fragment of a nucleic acid as used herein is a portion of the nucleic acid that is less than full-length. For example, for the present invention any length of nucleotide sequence that is less than the disclosed nucleotide sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, and SEQ ID NO:26 is considered to be a fragment. A fragment can also comprise at least a minimum length capable of hybridizing specifically with a native nucleic acid under stringent hybridization conditions as defined above. The length of such a minimal fragment is preferably at least 8 nucleotides, more preferably 15 nucleotides, even more preferably at least 20 nucleotides, and most preferably at least 30 nucleotides of a native nucleic acid sequence.

A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. "Primers" are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

Probes and primers are generally 11 nucleotides or more in length, preferably 18 nucleotides or more, more preferably 25 nucleotides, and most preferably 30 nucleotides or more. Such probes and primers hybridize specifically to a target DNA or RNA sequence under high stringency hybridization conditions and hybridize specifically to a target native sequence of another species under lower stringency conditions. Preferably, probes and primers according to the present invention have complete sequence similarity with the native sequence, although probes differing from the native sequence and that retain the ability to hybridize to target native sequences may be designed by conventional methods. Methods for preparing and using probes and primers are described, for example, in Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1–3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 (hereinafter, "Sambrook et al., 1989"); Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992); and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-

primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Primers and probes based on the native promoter sequences disclosed herein can be used to confirm and, if necessary, to modify the disclosed sequences by conventional methods, e.g., by re-cloning and re-sequencing.

Constructs and Expression Constructs

Native or synthetic nucleic acids according to the present invention can be incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a host cell. In a preferred embodiment, the nucleotide sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 or fragments, variants or derivatives thereof are incorporated into an expression cassette which includes the promoter regions of the present invention operably linked to a genetic component such as a selectable, screenable, or scorable marker gene.

In another embodiment, the disclosed nucleic acid sequences of the present invention as shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30 are operably linked to a genetic component such as a nucleic acid which confers a desirable characteristic associated with plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance. These genetic components such as marker genes or agronomic genes of interest can function in the identification of a transformed plant cell or plant, or a produce a product of agronomic utility.

In another embodiment, one genetic component produces a product which serves as a selection device and functions in a regenerable plant tissue to produce a compound which would confer upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to GUS (coding sequence for beta-glucuronidase), GFP (coding sequence for green fluorescent protein), LUX (coding gene for luciferase), antibiotic resistance marker genes, or herbicide tolerance genes. Examples of transposons and associated antibiotic resistance genes include the transposons Tns (bla), Tn5 (nptII), Tn7 (dhfr), penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline.

Characteristics useful for selectable markers in plants have been outlined in a report on the use of microorganisms (Advisory Committee on Novel Foods and Processes, July 1994). These include stringent selection with minimum number of nontransformed tissues, large numbers of independent transformation events with no significant interference with the regeneration, application to a large number of species, and availability of an assay to score the tissues for presence of the marker.

A number of selectable marker genes are known in the art and several antibiotic resistance markers satisfy these criteria, including those resistant to kanamycin (nptII), hygromycin B (aph IV) and gentamycin (aac3 and aacC4). Useful dominant selectable marker genes include genes encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin); and herbicide resistance genes (e.g., phosphinothricin acetyltransferase). A useful strategy for selection of transformants for herbicide resistance is described, e.g., in Vasil, Cell Culture and Somatic Cell Genetics of Plants, Vols. I–III, Laboratory Procedures and Their Applications Academic Press, New York, 1984. Particularly preferred selectable marker genes for use in the present invention would genes which confer resistance to compounds such as antibiotics like kanamycin, and herbicides like glyphosate (Della-Cioppa et al., Bio/Technology 5(6), 1987, U.S. Pat. No. 5,463,175, U.S. Pat. No. 5,633,435). Other selection devices can also be implemented and would still fall within the scope of the present invention.

For the practice of the present invention, conventional compositions and methods for preparing and using DNA constructs and host cells are employed, as discussed, inter alia, in Sambrook et al., 1989. In a preferred embodiment, the host cell is a plant cell. A number of DNA constructs suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; and R.R.D. Croy Plant Molecular Biology LabFax, BIOS Scientific Publishers, 1993. Plant expression constructs can include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences. They can also include a selectable marker as described to select for host cells containing the expression construct. Such plant expression constructs also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and a polyadenylation signal. Other sequences of bacterial origin are also included to allow the construct to be cloned in a bacterial host. The construct will also typically contain a broad host range prokaryotic origin of replication. In a particularly preferred embodiment, the host cell is a plant cell and the plant expression construct comprises a promoter region as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; an operably linked transcribable sequence; and a transcription termination sequence. Other regulatory sequences envisioned as genetic components in an expression construct include but is not limited to non-translated leader sequence which can be coupled with the promoter. In a particularly preferred embodiment, the host cell is a plant cell and the plant expression construct comprises a promoter region as disclosed in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NOS:27, SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30; an operably linked transcribable sequence, and a transcription termination sequence. Plant expression constructs also can comprise additional sequences including but not limited to polylinker sequences that contain restriction enzyme sites that are useful for cloning purposes.

Genetic Elements in Plant Expression Constructs

Plant expression constructs may include more than one expressible gene sequence, each operably linked to a dif-

ferent promoter. A number of promoters have utility for plant gene expression for any gene of interest including but not limited to selectable markers, scorable markers, genes for pest tolerance, disease tolerance, nutritional enhancements and any other gene of agronomic interest. Examples of constitutive promoters useful for plant gene expression include but are not limited to, the cauliflower mosaic virus (CaMV) P-35S promoter, which confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., Nature 313:810, 1985), including monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220:389, 1990); a tandemly duplicated version of the CaMV 35S promoter, the enhanced 35S promoter (P-e35S) the nopaline synthase promoter (An et al., Plant Physiol. 88:547, 1988), the octopine synthase promoter (Fromm et al., Plant Cell 1:977, 1989); and the figwort mosaic virus (P-FMV) promoter as described in U.S. Pat. No. 5,378,619 and an enhanced version of the FMV promoter (P-eFMV) where the promoter sequence of P-FMV is duplicated in tandem, the cauliflower mosaic virus 19S promoter, a sugarcane bacilliform virus promoter, a commelina yellow mottle virus promoter, and other plant DNA virus promoters known to express in plant cells.

A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals can be used for expression of an operably linked gene in plant cells, including promoters regulated by (1) heat (Callis et al., Plant Physiol. 88:965, 1988), (2) light (e.g., pea rbcS-3A promoter, Kuhlemeier et al., Plant Cell 1:471, 1989; maize rbcS promoter, Schaffner and Sheen, Plant Cell 3:997, 1991; or chlorophyll a/b-binding protein promoter, Simpson et al., EMBO J. 4:2723, 1985), (3) hormones, such as abscisic acid (Marcotte et al., Plant Cell 1:969, 1989), (4) wounding (e.g., wunI, Siebertz et al., Plant Cell 1:961, 1989); or (5) chemicals such as methyl jasmonate, salicylic acid, or Safener. It may also be advantageous to employ (6) organ-specific promoters (e.g., Roshal et al., EMBO J. 6:1155, 1987; Schernthaner et al., EMBO J. 7:1249, 1988; Bustos et al., Plant Cell 1:839, 1989). The promoters of the present invention are plant promoters that are capable of transcribing operably linked DNA sequences in rapidly growing meristematic tissue and reproductive tissues and can be operably linked to any gene of interest in an expression construct.

Plant expression constructs can include RNA processing signals, e.g., introns, which may be positioned upstream or downstream of a polypeptide-encoding sequence in the transgene. In addition, the expression constructs may include additional regulatory sequences from the 3'-untranslated region of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. USA 84:744 (1987); An et al., Plant Cell 1:115 (1989), e.g., a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions. 5' non-translated regions of a mRNA can play an important role in translation initiation and can also be a genetic component in a plant expression construct. For example, non-translated 5' leader sequences derived from heat shock protein genes have been demonstrated to enhance gene expression in plants (see, for example U.S. Pat. No. 5,362,865). These additional upstream and downstream regulatory sequences may be derived from a source that is native or heterologous with respect to the other elements present on the expression construct.

The promoter sequences of the present invention are used to control gene expression in plant cells. The disclosed promoter sequences are genetic components that are part of constructs used in plant transformation. The promoter sequences of the present invention can be used with any suitable plant transformation plasmid or construct containing a selectable or screenable marker and associated regulatory elements, as described, along with one or more nucleic acids expressed in a manner sufficient to confer a particular desirable trait. Examples of suitable structural genes of agronomic interest envisioned by the present invention would include but are not limited to one or more genes for insect tolerance, such as a *Bacillus thuringiensis* (B.t.) gene, pest tolerance such as genes for fungal disease control, herbicide tolerance such as genes conferring glyphosate tolerance, and genes for quality improvements such as yield, nutritional enhancements, environmental or stress tolerances, or any desirable changes in plant physiology, growth, development, morphology or plant product(s). For example, structural genes would include any gene that confers insect tolerance including but not limited to a Bacillus insect control protein gene as described in WO 9931248, herein incorporated by reference in its entirety, U.S. Pat. No. 5,689,052, herein incorporated by reference in its entirety, U.S. Pat. Nos. 5,500,365 and 5,880,275, herein incorporated by reference it their entirety. In another embodiment, the structural gene can confer tolerance to the herbicide glyphosate as conferred by genes including, but not limited to Agrobacterium strain CP4 glyphosate resistant EPSPS gene (aroA:CP4) as described in U.S. Pat. No. 5,633,435, herein incorporated by reference in its entirety, or glyphosate oxidoreductase gene (GOX) as described in U.S. Pat. No. 5,463,175, herein incorporated by reference in its entirety.

Alternatively, the DNA coding sequences can effect these phenotypes by encoding a non-translatable RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via antisense- or cosuppression-mediated mechanisms (see, for example, Bird et al., Biotech. Gen. Engin. Rev. 9:207,1991). The RNA could also be a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave a desired endogenous mRNA product (see for example, Gibson and Shillitoe, Mol. Biotech. 7:125, 1997). Thus, any gene which produces a protein or mRNA which expresses a phenotype or morphology change of interest is useful for the practice of the present invention.

In addition to regulatory elements or sequences located upstream (5') or within a DNA sequence, there are downstream (3') sequences that affect gene expression. Thus, the term regulatory sequence as used herein refers to any nucleotide sequence located upstream, within, or downstream to a DNA sequence which controls, mediates, or affects expression of a gene product in conjunction with the protein synthetic apparatus of the cell.

Those of skill in the art are aware of the constructs suitable for plant transformation. The promoter sequences of the present invention are preferably incorporated into an expression construct using screenable or scorable markers as described and tested in transient analyses to provide an indication of gene expression in transformed plants. Methods of testing gene expression in transient assays are known to those of skill in the art. Transient expression of marker genes has been reported using a variety of plants, tissues and DNA delivery systems. For example, types of transient analyses can include but are not limited to direct gene delivery via electroporation or particle bombardment of tissues in any transient plant assay using any plant species of interest. Such transient systems would include but are not limited to protoplasts from suspension cultures in wheat (Zhou et al., Plant Cell Reports 12:612. 1993), electropora-

tion of leaf protoplasts of wheat (Sethi et al., J. Crop Sci. 52: 152, 1983); electroporation of protoplast prepared from corn tissue (Sheen, J. The Plant Cell 3: 225, 1991), or particle bombardment of specific tissues of interest. The present invention encompasses the use of any transient expression system to evaluate regulatory sequences operatively linked to selected reporter genes, marker genes or agronomic genes of interest. Examples of plant tissues envisioned to test in transients via an appropriate delivery system would include but are not limited to leaf base tissues, callus, cotyledons, roots, endosperm, embryos, floral tissue, pollen, and epidermal tissue.

Any scorable or screenable marker can be used in a transient assay. Preferred marker genes for transient analyses of the promoters or 5' regulatory sequences of the present invention include a β-glucuronidase (GUS) gene or a green fluorescent protein (GFP) gene. The expression constructs containing the 5' regulatory sequences operably linked to a marker gene are delivered to the tissues and the tissues are analyzed by the appropriate mechanism, depending on the marker. The quantitative or qualitative analyses are used as a tool to evaluate the potential expression profile of the 5' regulatory sequences when operatively linked to genes of agronomic interest in stable plants. Ultimately, the 5' regulatory sequences of the present invention are directly incorporated into suitable plant transformation expression constructs comprising the 5' regulatory sequences operatively linked to a transcribable DNA sequence interest, transformed into plants and the stably transformed plants and progeny thereof analyzed for the desired expression profile conferred by the 5' regulatory sequences.

Suitable expression constructs for introducing exogenous DNA into plant cells would include but are not limited to disarmed Ti-plasmids for Agrobacterium-mediated methods. These constructs can contain a resistance marker, 1–2 T-DNA borders, and origins of replication for *E. coli* and Agrobacterium along with one or more genes of interest and associated regulatory regions. Those of skill in the art are aware that for Agrobacterium-mediated approaches a number of strains and methods are available. Such strains would include but are not limited to Agrobacterium strains C58, LBA4404, EHA101 and EHA105. Particularly preferred strains are *Agrobacterium tumefaciens* strains.

Exemplary nucleic acids which may be introduced by the methods encompassed by the present invention include, for example, DNA sequences or genes from another species, or even genes or sequences which originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. However, the term "exogenous" is also intended to refer to genes that are not normally present in the cell being transformed, or perhaps simply not present in the form, structure, etc., as found in the transforming DNA segment or gene, or genes which are normally present and that one desires to express in a manner that differs from the natural expression pattern, e.g., to over-express. Thus, the term "exogenous" gene or DNA is intended to refer to any gene or DNA segment that is introduced into a recipient cell, regardless of whether a similar gene may already be present in such a cell. The type of DNA included in the exogenous DNA can include DNA which is already present in the plant cell, DNA from another plant, DNA from a different organism, or a DNA generated externally, such as a DNA sequence containing an antisense message of a gene, or a DNA sequence encoding a synthetic or modified version of a gene.

The plant transformation constructs containing the promoter sequences of the present invention may be introduced into plants by any plant transformation method. Several methods are available for introducing DNA sequences into plant cells and are well known in the art. Suitable methods include but are not limited to bacterial infection (e.g., with Agrobacterium as described above), binary bacterial artificial chromosome constructs, direct delivery of DNA (e.g. via PEG-mediated transformation, desiccation/inhibition-mediated DNA uptake, electroporation, agitation with silicon carbide fibers), and acceleration of DNA coated particles (reviewed in Potrykus, Ann. Rev. Plant Physiol. Plant Mol. Biol., 42: 205, 1991).

Methods for specifically transforming dicots primarily use *Agrobacterium tumefaciens*. For example, transgenic plants reported include but are not limited to cotton (U.S. Pat. No. 5,004,863; U.S. Pat. No. 5,159,135; U.S. Pat. No. 5,518,908, WO 97/43430), soybean (U.S. Pat. No. 5,569, 834; U.S. Pat. No. 5,416,011; McCabe et al., Bio/Technology, 6:923, 1988; Christou et al., Plant Physiol., 87:671, 1988); Brassica (U.S. Pat. No. 5,463,174), and peanut (Cheng et al., Plant Cell Rep., 15: 653, 1996).

Similar methods have been reported in the transformation of monocots. Transformation and plant regeneration using these methods have been described for a number of crops including but not limited to asparagus (*Asparagus officinalis;* Bytebier et al., Proc. Natl. Acad. Sci. U.S.A., 84: 5345, 1987); barley (*Hordeum vulgarae;* Wan and Lemaux, Plant Physiol., 104: 37, 1994); maize (*Zea mays;* Rhodes, C. A., et al., Science, 240: 204, 1988; Gordon-Kamm, et al., Plant Cell, 2: 603, 1990; Fromm, et al., Bio/Technology, 8: 833, 1990; Koziel, et al., Bio/Technology, 11: 194, 1993); oats (*Avena sativa;* Somers, et al., Bio/Technology, 10: 1589, 1992); orchardgrass (*Dactylis glomerata;* Horn, et al., Plant Cell Rep., 7: 469, 1988); rice (*Oryza sativa*, including indica and japonica varieties, Toriyama, et al., Bio/Technology, 6: 10, 1988; Zhang, et al., Plant Cell Rep., 7: 379, 1988; Luo and Wu, Plant Mol. Biol. Rep., 6: 165, 1988; Zhang and Wu, Theor. Appl. Genet., 76: 835, 1988; Christou, et al., Bio/Technology, 9: 957, 1991); sorghum (*Sorghum bicolor;* Casas, A. M., et al., Proc. Natl. Acad. Sci. U.S.A., 90: 11212, 1993); sugar cane (Saccharum spp.; Bower and Birch, Plant J., 2: 409, 1992); tall fescue (*Festuca arundinacea;* Wang, Z. Y. et al., Bio/Technology, 10: 691, 1992); turfgrass (*Agrostis palustris;* Zhong et al., Plant Cell Rep., 13: 1, 1993); wheat (*Triticum aestivum;* Vasil et al., Bio/Technology, 10: 667, 1992; Weeks T., et al., Plant Physiol, 102: 1077, 1993; Becker, et al., Plant, J. 5: 299, 1994), and alfalfa (Masoud, S. A., et al., Transgen. Res., 5: 313, 1996). It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

Plant Analysis Methods

The transformed plants are analyzed for the presence of the genes of interest and the expression level and/or profile conferred by the promoter sequences of the present invention. Those of skill in the art are aware of the numerous methods available for the analysis of transformed plants. A variety of methods are used to assess gene expression and determine if the introduced gene(s) is integrated, functioning properly, and inherited as expected. For the present invention the promoters can be evaluated by determining the expression levels of genes to which the promoters are operatively linked. A preliminary assessment of promoter function can be determined by a transient assay method using reporter genes, but a more definitive promoter assessment can be determined from the analysis of stable plants. Methods for plant analysis include but are not limited to

Southern blots or northern blots, PCR-based approaches, biochemical analyses, phenotypic screening methods, field evaluations, and immunodiagnostic assays.

The methods of the present invention including but not limited to PCR technologies, genomic DNA isolation, expression construct construction, transient assays, and plant transformation methods are well known to those of skill in the art and are carried out using standard techniques or modifications thereof.

Glyphosate Spray Tests

In one embodiment a greenhouse or field evaluation for glyphosate tolerance is conducted. The term "glyphosate" is used herein to refer collectively to the parent herbicide N-phosphonomethylglycine (otherwise known as glyphosate acid), to a salt or ester thereof, or to a compound which is converted to N-phosphonomethylglycine in plant tissues or which otherwise provides N-phosphonomethylglycine in ionic form (otherwise known as glyphosate ion). Illustratively, water-soluble glyphosate salts useful herein are disclosed in U.S. Pat. No. 3,799,758 and No. 4,405,531 to Franz, the disclosure of which is incorporated herein by reference. Glyphosate salts that can be used according to the present invention include but are not restricted to alkali metal, for example sodium and potassium, salts; ammonium salt; C_{1-16} alkylammonium, for example dimethylammonium and isopropylammonium, salts; C_{1-16} alkanolammonium, for example monoethanolammonium, salt; C_{1-16} alkylsulfonium, for example trimethylsulfonium, salts; mixtures thereof and the like. The glyphosate acid molecule has three acid sites having different pKa values; accordingly mono-, di- and tribasic salts, or any mixture thereof, or salts of any intermediate level of neutralization, can be used.

Glyphosate salts are commercially significant in part because they are water-soluble. Many ammonium, alkylammonium, alkanolammonium, alkylsulfonium and alkali metal salts are highly water-soluble, allowing for formulation as highly concentrated aqueous solutions which can be diluted in water at the point of use.

Such concentrated aqueous solutions can contain about 50 to about 500 grams per liter of glyphosate, expressed as acid equivalent (g a.e./l). Higher glyphosate concentrations, for example about 300 to about 500 g a.e./l, are preferred.

Glyphosate salts are alternatively formulated as water-soluble or water-dispersible compositions, in the form for example of powders, granules, pellets or tablets. Such compositions are often known as dry formulations, although the term "dry" should not be understood in this context to imply the complete absence of water. Typically, dry formulations contain less than about 5% by weight of water, for example about 0.5% to about 2% by weight of water. Such formulations are intended for dissolution or dispersion in water at the point of use.

Contemplated dry glyphosate formulations can contain about 5% to about 80% by weight of glyphosate, expressed as acid equivalent (% a.e.). Higher glyphosate concentrations within the above range, for example about 50% to about 80% a.e., are preferred. Especially useful salts of glyphosate for making dry formulations are sodium and ammonium salts.

Plant treatment compositions and liquid and dry concentrate compositions of the invention can optionally contain one or more desired excipient ingredients. Especially useful excipient ingredients for glyphosate compositions are surfactants, which assist in retention of aqueous spray solutions on the relatively hydrophobic surfaces of plant leaves, as well as helping the glyphosate to penetrate the waxy outer layer (cuticle) of the leaf and thereby contact living tissues within the leaf. Surfactants can perform other useful functions as well.

There is no restriction in the type or chemical class of surfactant that can be used in glyphosate compositions of the invention. Nonionic, anionic, cationic and amphoteric types, or combinations of more than one of these types, are all useful in particular situations. However, it is generally preferred that at least one of the surfactants, if any, present should be other than anionic, i.e., at least one of the surfactants should be nonionic, cationic or amphoteric.

Many surfactants useful herein have a chemical structure that comprises one or more moieties each consisting of a single C_{2-4} alkylene oxide unit or a polymerized or copolymerized chain of C_{2-4} alkylene oxide units. Such surfactants are referred to as polyoxyalkylene surfactants and include nonionic, anionic, cationic and amphoteric types. Polyoxyalkylene surfactants useful in presently contemplated compositions contain about 2 to about 100 C_{2-4} alkylene oxide units. In preferred polyoxyalkylene surfactants the alkylene oxide units form one or more chains of either ethylene oxide or copolymerized ethylene oxide and propylene oxide, each chain of alkylene oxide units having a terminal hydrido group or a C_{1-4} alkyl or C_{1-4} alkanoyl end-cap.

Hydrophobic moieties of surfactants useful in compositions of the invention can be essentially hydrocarbon based, in which case the hydrophobic moieties are typically C_{8-24}, preferably C_{12-18}, alkyl, alkenyl, alkylaryl, alkanoyl or alkenoyl chains. These chains can be linear or branched. Alternatively, the hydrophobic moieties can contain silicon atoms, for example in the form of siloxane groups such as heptamethyltrisiloxane groups, or fluorine atoms, for example as partially-fluorinated alkyl or perfluoroalkyl chains.

Among nonionic surfactants, especially preferred classes include polyoxyethylene alkyl, alkenyl or alkylaryl ethers, such as ethoxylated primary or secondary alcohols or alkylphenols, polyoxyethylene alkyl or alkenyl esters, such as ethoxylated fatty acids, polyoxyethylene sorbitan alkyl esters, glyceryl alkyl esters, sucrose esters, alkyl polyglycosides, and the like. Representative specific examples of such nonionic surfactants include polyoxyethylene (9) nonylphenol, Neodol™ 25-7 of Shell (a polyoxyethylene (7) C_{12-15} linear primary alcohol), Tergitol™ 15-S-9 of Union Carbide (a polyoxyethylene (9) C_{12-15} secondary alcohol), Tween™ 20 of ICI (a polyoxyethylene (20) sorbitan monolaurate) and Agrimul™ PG-2069 of Henkel (a C_{9-11} alkyl polyglucoside).

Among cationic surfactants, especially preferred classes include polyoxyethylene tertiary alkylamines or alkenylamines, such as ethoxylated fatty amines, quaternary ammonium surfactants, polyoxyethylene alkyletheramines, and the like. Representative specific examples of such cationic surfactants include polyoxyethylene (5) cocoamine, polyoxyethylene (15) tallowamine, distearyldimethylammonium chloride, cetyltrimethylammonium bromide, methyl bis(2-hydroxyethyl)cocoammonium chloride, N-dodecylpyridine chloride and polyoxypropylene (8) ethoxytrimethylammonium chloride. Particularly preferred polyoxyethylene alkyletheramines are those disclosed in PCT Publication No. WO 96/32839. Many cationic quaternary ammonium surfactants of diverse structures are known in the art to be useful in combination with glyphosate and can be used in compositions contemplated herein; such quaternary ammonium surfactants have the formula

$$(NR^aR^bR^cR^d)_mA_n$$

where A is a suitable anion such as chloride, bromide, iodide, acetate, sulfate or phosphate, m and n are integers such that the positive electrical charges on cations ($NR^aR^bR^cR^d$) balance the negative electrical charges on anions A, and options for R^a, R^b, R^c and R^d include, without limitation:

(i) R^a is benzyl or C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, and R^b, R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

(ii) R^a and R^b are independently C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, and R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

(iii) R^a is C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, R^b is a polyoxyalkylene chain having about 2 to about 100 C_{2-4} alkylene oxide units, preferably ethylene oxide units, and R^c and R^d are independently C_{1-4} alkyl, preferably methyl;

(iv) R^a is C_{8-24}, preferably C_{12-18}, alkyl or alkenyl, R^b and R^c are polyoxyalkylene chains having in total about 2 to about 100 C_{2-4} alkylene oxide units, preferably ethylene oxide units, and R^d is C_{1-4} alkyl, preferably methyl; or

(v) R^a is a polyoxyalkylene chain having about 2 to about 100 C_{2-4} alkylene oxide units in which C_{3-4} alkylene oxide units, preferably propylene oxide units, predominate and R^b, R^c and R^d are independently C_{1-4} alkyl, preferably methyl or ethyl.

Particularly preferred quaternary ammonium surfactants of this type are those disclosed in U.S. Pat. No. 5,464,807 to Claude et al.

In one embodiment, the anion A associated with such a quaternary ammonium surfactant can be a glyphosate anion.

Among amphoteric surfactants, including as is customary in the art surfactants more correctly described as zwitterionic, especially preferred classes include polyoxyethylene alkylamine oxides, alkylbetaines, alkyl-substituted amino acids and the like. Representative examples of such amphoteric surfactants include dodecyldimethylamine oxide, polyoxyethylene (2) cocoamine oxide and stearyldimethylbetaine.

Standard reference sources from which one of skill in the art can select suitable surfactants, without limitation to the above mentioned classes, include *Handbook of Industrial Surfactants,* Second Edition (1997) published by Gower, *McCutcheon's Emulsifiers and Detergents,* North American and International Editions (1997) published by MC Publishing Company, and *International. Cosmetic Ingredient Dictionary,* Sixth Edition (1995) Volumes 1 and 2, published by the Cosmetic, Toiletry and Fragrance Association.

Other optional components of compositions of the invention include agents to modify color, viscosity, gelling properties, freezing point, hygroscopicity, caking behavior, dissolution rate, dispersibility, or other formulation characteristics.

Examples of commercial formulations of glyphosate include, without restriction, those sold by Monsanto Company as ROUNDUP®, ROUNDUP® ULTRA, ROUNDUP® CT, ROUNDUP® EXTRA, ROUNDUP® BIACTIVE, ROUNDUP® BIOFORCE, RODEO®, POLARIS®, SPARK® and ACCORD® herbicides, all of which contain glyphosate as its isopropylammonium salt; those sold by Monsanto Company as ROUNDUP® DRY and RIVAL® herbicides, which contain glyphosate as its ammonium salt; that sold by Monsanto Company as ROUNDUP® GEOFORCE, which contains glyphosate as its sodium salt; and that sold by Zeneca Limited as TOUCHDOWN® herbicide, which contains glyphosate as its trimethylsulfonium salt.

The selection of application rates for a glyphosate formulation that are biologically effective is within the skill of the ordinary agricultural technician One of skill in the art will likewise recognize that individual plant conditions, weather conditions and growing conditions can affect the results achieved in practicing the process of the present invention. Over two decades of glyphosate use and published studies relating to such use have provided abundant information from which a weed control practitioner can select glyphosate application rates that are herbicidally effective on particular species at particular growth stages in particular environmental conditions.

A process of the present invention is applicable to any and all plant species on which glyphosate is biologically effective as a herbicide or plant growth regulator. This encompasses a very wide variety of plant species worldwide. Likewise, compositions of the invention can be applied to any and all plant species on which glyphosate is biologically effective.

In one embodiment, a glyphosate-containing herbicide is applied to the plant comprising the DNA constructs of the present invention, and the plants are evaluated for tolerance to the glyphosate herbicide. Any formulation of glyphosate can be used for testing plants comprising the DNA constructs of the present invention. For example, a glyphosate composition such as Roundup Ultra™ can be used. The testing parameters for an evaluation of the glyphosate tolerance of the plant will vary depending on a number of factors. Factors would include, but are not limited to the type of glyphosate formulation, the concentration and amount of glyphosate used in the formulation, the type of plant, the plant developmental stage during the time of the application, environmental conditions, the application method, and the number of times a particular formulation is applied. For example, plants can be tested in a greenhouse environment using a spray application method. The testing range using Roundup Ultra™ can include, but is not limited to 8 oz/acre to 256 oz/acre. The preferred commercially effective range can be from 16 oz/acre to 64 oz/acre of Roundup Ultra™, depending on the crop and stage of plant development. A crop can be sprayed with at least one application of a glyphosate formulation. For testing in cotton an application of 32 oz/acre at the 3-leaf stage may be followed by additional applications at later stages in development. For wheat an application of 32 oz/acre of Roundup Ultra™ at the 3–5 leaf stage can be used and may be followed with a pre- or post-harvest application, depending on the type of wheat to be tested. The test parameters can be optimized for each crop in order to find the particular plant comprising the constructs of the present invention that confers the desired commercially effective glyphosate tolerance level.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

EXAMPLES

Example 1

The plasmid constructs used are either pUC cloning constructs or double border plant transformation constructs

containing an *E. coli* origin of replication such as ori322, a broad host range origin of replication such as oriV or oriRi, and a coding region for a selectable marker such as Spc/Str that encodes for Tn7 aminoglycoside adenyltransferase (aadA) confers resistance to spectinomycin or streptomycin, or a gentamicin (Gm, Gent) selectable marker. For plant transformation, the host bacterial strain was *Agrobacterium tumefaciens* ABI or LBA4404.

The genetic elements are described as follows: P-e35S is the 35S RNA from CaMV containing a duplication of the −90–300 region as described in U.S. Pat. No. 5,424,200 herein incorporated by reference in its entirety; P-FMV is the 34S promoter from Figwort Mosaic Virus as described in U.S. Pat. No. 5,378,619 herein incorporated by reference in its entirety; P-eFMV is a derivative of the FMV promoter containing a duplicated FMV promoter; CTP2 is the transit peptide region of Arabidopsis EPSP synthase as described in U.S. Pat. No. 5,633,435; aroA:CP4syn (aroA:CP4) is the coding region for CP4 EPSP (synthetic sequence) as described in U.S. Pat. No. 5,633,435 or further modified for expression in plants based on codon usage of particular plant species; E9 3' is the 3' end of an isolate of the pea RbcS gene that functions as a polyadenylation signal; nos is the 3' end of the nopaline synthase gene that functions as a polyadenylation signal; Hsp70 is the non-translated leader sequence from *Petunia hybrida* as described in U.S. Pat. No. 5,362,865 herein incorporated by reference in its entirety; GUS is the beta-glucuronidase coding sequence from *E. coli* (Jefferson, R. A. *Proc. Natl. Acad. Sci. U.S.A.*, 83:8447–8451, 1987); the right border (RB) and left borders (LB) are from the Ti plasmid of *Agrobacterium tumefaciens* octopine and nopaline strains. The P-AtAct2 is the promoter from the *Arabidopsis thaliana* actin 2 gene; AtAct2i is the intron in the 5' untranslated region (UTR) of the *Arabidopsis thaliana* actin 2 gene; P-AtAct8 is the promoter from the *Arabidopsis thaliana* actin 8 gene; AtAct2i is the intron in the 5' UTR of the *Arabidopsis thaliana* actin 8 gene; P-AtAct11 is the promoter from the *Arabidopsis thaliana* actin 11 gene; AtAct11i is the intron in the 5' UTR of the *Arabidopsis thaliana* actin 11 gene; P-AtAct1 a is the promoter from the *Arabidopsis thaliana* actin 1a gene, L-AtAct1 a is the untranslated leader and I-AtAct1a is the intron from the genomic DNA of the actin 1a gene; P-AtAct1b is the promoter from the *Arabidopsis thaliana* actin 1b gene, L-AtAct1b is the untranslated leader and I-AtAct1b is the intron from the genomic DNA of the actin 1b gene; P-AtAct3 is the promoter from the *Arabidopsis thaliana* actin 3 gene, L-AtAct3 is the untranslated leader and I-AtAct3 is the intron from the genomic DNA of the actin 3 gene; P-AtAct7 is the promoter from the *Arabidopsis thaliana* actin 7 gene, L-AtAct7 is the untranslated leader and I-AtAct7 is the intron from the genomic DNA of the actin 7 gene; P-AtAct12 is the promoter from the *Arabidopsis thaliana* actin 12 gene, L-AtAct12 is the untranslated leader and I-AtAct12 is the intron from the genomic DNA of the actin 12 gene; P-AtEF1α (P-AtEF1 or EF1α) is the promoter from the *Arabidopsis thaliana* elongation factor gene 1α, AtEF1α-i (AtEF1-i) is the intron in the 5' UTR of the *Arabidopsis thaliana* elongation factor gene 1α.

FIGS. **1–18** provide examples of plant transformation constructs that contain one to three plant expression cassettes. Multiple combinations of plant expression cassettes comprising the promoter and genetic elements of the present invention can be constructed and tested in crops plants by those skilled in the art of plant molecular biology without undue experimentation. The constructs illustrated in the Figures are not to be construed as the only constructs that can be assembled, but serve only as examples to those skilled in the art. FIG. **1** (pCGN8086) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct8) operably linked to a gene of interest (CTP2-aroA:CP4syn). FIG. **2** (pMON45325) provides an example of a plant transformation construct containing two expression cassettes comprising at least one promoter of the present invention (P-AtAct11) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **3** (pMON45331) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtEF1 plus intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **4** (pMON45332) provides an example of a plant transformation construct containing two expression cassettes comprising at least one promoter of the present invention (P-AtEF1 plus intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **5** (pMON9190) provides an example of a plant transformation construct containing three expression cassettes wherein at least two promoters of the present invention (P-AtEF1 plus intron, AtEF1α-i; P-AtAct2 plus intron, AtAct2i) are operably linked to at least one gene of interest (CTP2-aroA:CP4syn) and the P-eFMV promoter operably lined to CTP2-aroA:CP4syn. FIG. **6** (pMON9153) plant expression cassettes are identical to those illustrated in FIG. **4** (pMON45332), this plasmid map is illustrated for the purpose of identification of the expression cassettes for data shown on plant phenotype in the data tables shown in the specification. FIG. **7** (pCGN8099) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-FMV-AtEF1α and P-e35S-AtAct8, driving transcription of the gene of interest (aroA:CP4syn). FIG. **8** (pCGN8088) provides an example of a plant transformation construct containing two expression cassettes comprising one promoter of the present invention, P-AtAct8 plus intron, AtAct8i, and the P-eFMV promoter driving expression of a gene of interest (aroA:CP4syn). FIG. **9** (pCGN8068) provides an example of a plant transformation construct containing two expression cassettes comprising one promoter of the present invention, P-AtAct2 plus intron, AtAct2i, and the P-eFMV promoter driving expression of a gene of interest (aroA:CP4syn). FIG. **10** (pCGN8096) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-FMV/AtAct11 and P-e35S-AtAct2, driving transcription of the gene of interest (aroA: CP4syn). FIG. **11** (pCGN9151) provides an example of a plant transformation construct containing two expression cassettes comprising hybrid promoters of the present invention, P-FMV-AtEF1α and P-e35S-AtAct2, driving transcription of the gene of interest (aroA:CP4syn). FIG. **12** (pMON10156) provides an example of a plant transformation construct containing one expression cassette comprising the P-eFMV promoter driving expression of the aroA:CP4syn gene of interest, this vector is used for comparative purposes with the promoter sequences of the present invention. FIG. **13** (pMON52059) provides an example of a plant transformation construct containing one expression cassette comprising a hybrid promoter (P-FMV-AtEF1α) driving the expression of the gene of interest (aroA:CP4syn). FIG. **14** (pMON54952) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct1a plus AtAct1a intron) operably linked to at least one gene of interest (CTP2-

aroA:CP4syn). FIG. **15** (pMON54953) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct1b plus AtAct1b intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **16** (pMON54954) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct3 plus AtAct3 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **17** (pMON54955) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct7 plus AtAct7 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn). FIG. **18** (pMON54956) provides an example of a plant transformation construct containing one expression cassette comprising one promoter of the present invention (P-AtAct12 plus AtAct12 intron) operably linked to at least one gene of interest (CTP2-aroA:CP4syn).

Example 2

The cloning constructs and GUS constructs are listed in Table 1. The Arabidopsis actin 2 promoter and intron (Genbank accession number U41998 as described in An et al., *Plant J.* 10: 107–121, 1996) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template (Rogers and Bendich, *Plant Mol. Biol.* 5:69, 1998) using SEQ ID NO:1 (forward primer) and SEQ ID NO:2 (reverse primer) in a reaction as follows: 0.5 μg template DNA, 25 pmole of each primer, taq polymerase (BMB, Indianapolis, Ind.) using wax beads for "hot start" PCR. The PCR thermocycler conditions were as follows: 94° C. for one minute; 30 cycles of: 92° C. for 40 seconds, 55° C. for one minute, 72° C. for one minute and 30 seconds; and a five minute 72° C. extension. The PCR reaction was purified using GeneClean II (Bio101 Inc., Vista, Calif.), digested with HindIII and NcoI, and ligated into construct pMON26149 (Table 1) digested with HindIII and NcoI. The promoter clone was sequence verified and the resulting construct was designated pMON26170 (Table 1).

TABLE 1

Cloning Constructs and GUS Constructs containing Arabidopsis Actin and EF1 promoter sequences

Construct	Description	Promoter*/Gene/3'
pMON26149	cloning construct	
pMON26170	plant expression construct	Act2/GUS/nos
pMON26171	plant expression construct	Act8/GUS/nos
pMON8677	cloning construct	
pMON48407	plant expression construct	Act11/GUS/nos
pMON26152	cloning construct	
pMON26177	plant expression construct	EF1/GUS/nos
pMON11750	plant expression construct	e35S/GUS/nos
pMON15737	plant expression construct	FMV/GUS/nos

*the actin and elongation factor promoter sequences also contain the intron sequence from the 5' UTR of the corresponding gene.

Example 3

The Arabidopsis actin 8 promoter and intron (Genbank accession number U42007 as described in An et al., *Plant J.* 10:107–121, 1996) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:3 (forward primer) and SEQ ID NO:4 (reverse primer). The promoter was cloned using restriction enzymes as described in Example 2, sequence verified, and the resulting construct was designated pMON26171 (Table 1).

Example 4

The Arabidopsis actin 11 promoter and intron (Genbank accession number U27981 as described in Huang et al., *Plant Mol. Biol.*, 33:125–139, 1997) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:5 (forward primer) and SEQ ID NO:6 (reverse primer). The promoter was cloned using restriction enzymes EcoRV and NcoI and ligated into pMON8677 (Table 1), sequence verified, and the resulting construct was designated pMON48407 (Table 1).

Example 5

The Arabidopsis elongation factor 1α (AtEF1α) promoter and intron (Genbank accession number X16430 as described in Axelos et al., *Mol. Gen. Genet.* 219:106–112, 1989; Curie et al., *NAR* 19:1305–1310; Curie et al., *Plant Mol. Biol.* 18:1083–1089, 1992; Curie et al., *Mol. Gen. Genet.* 238:428–436, 1993) was isolated using *Arabidopsis thaliana* Landsberg erecta DNA as a template PCR conditions and purification methods described in Example 2 using primers SEQ ID NO:7 (forward primer) and SEQ ID NO:8 (reverse primer). The promoter was cloned using restriction enzymes HindIII and NcoI and ligated into pMON26152 (Table 1) as described in Example 2, sequence verified, and the resulting construct was designated pMON26177 (Table 1).

Example 6

The plant transformation constructs described were mated into Agrobacterium. Cotton transformation was performed essentially as described in WO/0036911, herein incorporated by reference in its entirety. The Arabidopsis transformation was performed as described in Ye et al., *Plant Journal* 19:249–257, 1999. The tomato transformation was performed as described in U.S. Pat. No. 5,565,347 herein incorporated by reference in its entirety.

Example 7

A DNA construct is transformed into a target crop of interest via an appropriate delivery system such as an Agrobacterium-mediated transformation method (see for example U.S. Pat. No. 5,569,834 herein incorporated by reference in its entirety, U.S. Pat. No. 5,416,011 herein incorporated by reference in its entirety, U.S. Pat. No. 5,631,152 herein incorporated by reference in its entirety, U.S. Pat. No. 5,159,135 herein incorporated by reference in its entirety, U.S. Pat. No. 5,004,863 herein incorporated by reference in its entirety, and U.S. Provisional Appln. No. 60/111795 herein incorporated by reference in its entirety. Alternatively, a particle bombardment method may be used (see for example Patent Applns. WO 92/15675. WO 97/48814 and European Patent Appln. 586,355, and U.S. Pat. Nos. 5,120,657, 5,503,998, 5,830,728 and 5,015,580, all of which are herein incorporated by reference in their entirety).

A large number of transformation and regeneration systems and methods are available and well-known to those of

skill in the art. The stably transformed plants and progeny are subsequently analyzed for expression of the gene in tissues of interest by any number of molecular, immunodiagnostic, biochemical, and/or field evaluation methods known to those of skill in the art, including, but not limited to a spray test with a glyphosate formulation at commercially effective concentrations performed in a growth chamber or field environment.

Example 8

The GUS assays are performed by routine methods known to those of skill in the art (see for example, Jefferson et al., *EMBO J.* 6:3901, 1987). For cotton, RO plants were tested. The tissue was size selected at various stages in development, samples and pooled for analysis. The cotton floral bud was harvested and the male reproductive tissue samples (anthers and filaments), female reproductive tissue samples (entire stigma, style, and ovary), and corolla (sepals and petals) were taken. For the size selection, three floral buds from each stage were selected that included several sizes including small (less than 0.5 cm), medium (from 0.5–0.7 cm), and large (candle stage or open flower). Leaf samples were collected about 1–2 weeks after the cotton plants were placed in the greenhouse, and the other samples were collected approximately 1–2 months later. The first flowers were not collected (the first five fruiting positions were left intact).

For Arabidopsis, V1 plants were analyzed and only homozygous and heterozygous segregants were tested Eight to ten events per construct were analyzed (five plants per event).

The GUS results for Arabidopsis represent pooled samples of 8–10 events. The values in the disclosed tables (Table 2 and Table 3) represent the average GUS expression for the designated tissue (pmol/MU/min/mg).

Example 9

Plants were analyzed for GUS expression in leaf tissue and reproductive tissues including immature floral buds and flowers. The results are shown in Table 2. Constructs tested included pMON48407 (P-AtAct11+intron/GUS/nos), pMON26170 (P-AtAct2+intron/GUS/nos), pMON26171 (P-AtAct8+intron/GUS/nos), pMON11750 (e35S/GUS/nos), pMON26177 (P-EF1α+intron/GUS/nos), and pMON15737 (P-FMV/GUS/nos). The actin and elongation factor promoters conferred high levels of GUS expression in multiple tissues including reproductive tissues.

TABLE 2

Average Arabidopsis V1 GUS Expression

Construct	Leaf	Immature Floral Bud	Flower	Gynoecium	Androecium
pMON48407	6944	7394	8359	ND	ND
pMON26170	45238	74099	54502	73623	217292
pMON26171	29343	35884	37125	76311	207100
pMON11750	60844	14032	16263	35882	115049
pMON26177	47598	72871	96420	191066	507370
pMON15737	28314	57903	84457	44696	87876

Example 10

The RO cotton plants were tested for expression of the GUS reporter gene in selected tissues of various stages of development. The floral buds were staged by size (small, medium, and large; large=candle and open flower). The androecium represented the male reproductive tissues including the entire receptacle (stigma, style, and ovaries). The corolla sample was composed of sepals and petals. The tissue was prepared and GUS assays performed as described in EXAMPLE 8. The results are summarized in Table 3. The constructs tested included pMON48407 (P-EF1α+intron/gus/nos), pMON26170 (P-AtAct2+intron/gus/nos), and pMON48407 (P-AtAct11+intron/gus/nos).

Six plants were tested and average GUS values obtained for pMON26177. Twenty plants were tested and average GUS values obtained for for pMON26170. Eight plants were tested and average GUS values obtained for pMON48407. The results demonstrate that the actin and elongation factor promoters can be used for effective expression of operably linked genes, particularly in reproductive tissues

TABLE 3

GUS Assay Results for Cotton Plants

Construct	Promoter/intron	Tissue Tested	GUS Results
pMON26177	EF1α	Leaf	11600
pMON26177	EF1α	Small Corolla	396
pMON26177	EF1α	Small Gynoecium	8670
pMON26177	EF1α	Small Androecium	13771
pMON26177	EF1α	Medium Corolla	362
pMON26177	EF1α	Medium Gynoecium	3318
pMON26177	EF1α	Medium Androecium	8006
pMON26177	EF1α	Large Corolla	351
pMON26177	EF1α	Large Gynoecium	500
pMON26177	EF1α	Large Androecium	15512
pMON26170	Act2	Leaf	12718
pMON26170	Act2	Small Corolla	1296
pMON26170	Act2	Small Gynoecium	16684
pMON26170	Act2	Small Androecium	7570
pMON26170	Act2	Medium Corolla	742
pMON26170	Act2	Medium Gynoecium	10041
pMON26170	Act2	Medium Androecium	7893
pMON26170	Act2	Large Corolla	289
pMON26170	Act2	Large Gynoecium	3218
pMON26170	Act2	Large Androecium	42737
pMON48407	Act11	Leaf	28289
pMON48407	Act11	Small Corolla	10
pMON48407	Act11	Small Gynoecium	40755
pMON48407	Act11	Small Androecium	47834
pMON48407	Act11	Medium Corolla	742
pMON48407	Act11	Medium Gynoecium	52495
pMON48407	Act11	Medium Androecium	35573
pMON48407	Act11	Large Corolla	1072

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TABLE 3-continued

GUS Assay Results for Cotton Plants

Construct	Promoter/intron	Tissue Tested	GUS Results
pMON48407	Act11	Large Gynoecium	4869
pMON48407	Act11	Large Androecium	42737

Example 11

Transformed plants were also tested in a greenhouse spray test using Roundup Ultra™ a glyphosate formulation with a Track Sprayer device (Roundup Ultra is a registered trademark of Monsanto Company). Plants were at the "two" true leaf or greater stage of growth and the leaves were dry before applying the Roundup® spray. The formulation used was Roundup Ultra™ as a 3 lb/gallon a.e. (acid equivalent) formulation. The calibration used was as follows:

For a 20 gallons/Acre spray volume:
Nozzle speed: 9501 evenflow
Spray pressure: 40 psi
Spray height 18 inches between top of canopy and nozzle tip
Track Speed 1.1 ft/sec., corresponding to a reading of 1950–1.0 volts.

Formulation: Roundup Ultra™ (3 lbs. A.e./gallon)

The spray concentrations will vary, depending on the desired testing ranges. For example, for a desired rate of 8 oz/acre a working solution of 3.1 ml/L is used, and for a desired rate of 64 oz/A a working range of 24.8 ml/L is used.

The evaluation period will vary, depending on the crop, stage of plant development, and tolerance level desired.

Example 12

The plant expression constructs used for tomato transformation are listed in Table 4. Tomato plants (T0) containing constructs comprising at least one actin or elongation factor promoter (with intron) operably linked to an aroA:CP4 glyphosate tolerance gene are screened in a greenhouse glyphosate spray test with glyphosate (Roundup Ultra™) formulation for the efficiency of conferring glyphosate tolerance to transgenic tomato plants. Optionally, at least one actin or elongation factor promoter sequence operably linked to an aroA:CP4 gene and an eFMV caulimovirus promoter operably linked to an aroA:CP4 transformed into tomato plants are screened by spray application with glyphosate (Roundup Ultra™). Tomato plants are sprayed with 48 oz./acre then evaluated at two weeks post application for analysis of vegetative tolerance and up to 60 days post-application for analysis of reproductive tolerance. The results are shown in Table 4 and ranked according to efficiency of selecting reproductive tolerant lines. The percent vegetative tolerance is the percentage of the lines screened that demonstrated sufficient vegetative tolerance to glyphosate damage to be considered for further studies of agronomic traits in preparation for commercially candidacy. The percent reproductive tolerance is the percentage of the vegetative tolerant lines that also demonstrated sufficient reproductive tolerance to be considered for further agronomic evaluation. All of the constructs proved functional for providing vegetative tolerance and reproductive tolerance to the transgenic tomato plants. Various combinations of promoters are able to increase the efficiency at which vegetative and reproductive tolerant lines could be selected by screening in this experiment. Constructs containing the Arabidopsis EF1α promoter are more specifically associated with a high percentage of vegetatively tolerant lines. P-Act2 promoter in combination with P-eFMV and P-AtEF1α (pCGN9190) provided an increase in the percentage of reproductively tolerant lines that are screened by this method.

TABLE 4

Greenhouse Track Spray Trials with Application Rate of 48 oz./Acre*

Construct	Description	# Lines Tested	% Vegetative Tolerance[1]	% Reprod. Toler.[2]
pCGN9190	eFMV/CP4 + EF1α/CP4 + Act2/CP4	930	83.2	52.4
pCGN9153	EF1α/CP4 + eFMV/CP4	391	73.9	38.9
pCGN8086	Act8/CP4	21	47.6	38.1
pCGN8099	FMV-EF1α/CP4 + Act8/CP4	71	84.5	36.6
pCGN8088	eFMV/CP4 + Act8/CP4	144	79.9	34.7
pMON45325	eFMV/CP4 + Act11/CP4	90	70.0	34.4
pCGN8096	FMV-Act11/CP4 + Act2/CP4	201	62.7	10.4
pCGN8067	Act2/CP4	205	67.3	8.8

*one application
[1]Pooled Results from 25 screens. Scored 14 days post-application
[2]Pooled Results from 25 screens. Scored up to 60 days post-application

Tomato seed yield is used as a measure of the efficacy of the various promoter sequences and combination of expression cassettes used in the present invention for conferring glyphosate tolerance to transgenic tomato plants. In Table 5, the results of three field experiments are shown on transgenic tomato plants containing constructs with the promoters of the present invention driving expression of the aroA:CP4 coding sequence for glyphosate tolerance. Experiment 1 is a test of the plants produced from the constructs that contain the Figwort mosaic virus promoter (P-FMV) in the native and the duplicated version (P-eFMV) and additional genetic elements in the constructs that are also found in the constructs used to test the promoter sequences of the present invention. Additional genetic elements such as the source of the 5' untranslated sequence and the chloroplast transit peptide are also tested. The construct pMON20998 comprises the P-eFMV, linked to the petunia Hsp70 5' UTR, leader linked to the Arabidopsis EPSPS chloroplast transit peptide (CTP2), linked to the E9 3' termination region. The construct pMON20999 differs from pMON20998 only in that the promoter is P-FMV. The construct pMON10156 differs from pMON20998 only in that the CTP is from the

Petunia EPSPS chloroplast transit peptide (CTP4). The construct pMON45312 differs from pMON20998 only in that the leader sequence is the native FMV leader sequence.

Tomato plants are transplanted into the field in rows. The plants are spray treated in the field at a rate of 48 oz./Acre with Roundup herbicide. The tomato seed is collected from the fruit and weighted. An unsprayed tomato line serves as the control for comparison purposes and the efficacy of each construct is expressed as a percentage of the control. The result of Experiment 1 (column 1 of Table 5) is that the FMV promoter and P-eFMV only provide 5–11% of the seed production of an unsprayed check. Experiment 2, and 3 tests the constructs of the present invention at different locations (columns 2 and 3 of Table 5). Experiment 2 is conducted at the same location as Experiment 1, the constructs pCGN8099 (FIG. 7), pCGN9151 (FIG. 11) and pCGN9190 (FIG. 5) performed well by providing 25–46% of the seed relative to an unsprayed check. At a different location that has a cooler growing season, Experiment 3 demonstrated that pCGN8068 (FIG. 9), pCGN8088 (FIG. 8), pCGN8099, pCGN9151, pCGN9153 (FIG. 6), and pMON45325 (FIG. 2) are able to confer sufficient glyphosate tolerance for the tomatoes to set 34–77% of normal seed set relative to an unsprayed check.

TABLE 5

Tomato seed yield experiments

	Exp. 1 Seed wt grams	% of Control	Exp. 2 Seed wt grams	% of Control	Exp. 3 Seed wt grams	% of Control
pMON20998	0.52	5.3				
pMON20999	0.84	8.6				
pMON10156	0.50	5.1				
pMON45312	1.07	11.0				
pCGN8068			0.48	8.4	7.06	77.8
pCGN8088			0.43	7.6	3.09	34.1
pCGN8096			0.40	7.0		
pCGN8099			1.85	32.5	6.93	76.4
pCGN9151			1.46	25.7	6.11	67.4
pCGN9153			0.68	12.0	4.03	44.4
pCGN9190			2.64	46.4		

TABLE 5-continued

Tomato seed yield experiments

	Exp. 1 Seed wt grams	% of Control	Exp. 2 Seed wt grams	% of Control	Exp. 3 Seed wt grams	% of Control
pMON45325			0.31	5.4	3.37	37.2
pCGN8067						
Control	9.73	100.0	5.69	100.0	9.07	100.0

Example 13

SEQ ID NOS: 1–8, and SEQ ID NOS: 13–21 are PCR primers designed from publicly available sequence information for *Arabidopsis thaliana* Act1, Act2 (Genbank #U41998), Act3, Act7, Act8 (Genbank #ATU42007), Act11 (Genbank #ATU27981), Act12 and Elf1α (Genbank #X164:30) genes. These sequences are used to extend the nucleic acid sequence using polymerase chain reaction (PCR) amplification techniques (see for example, Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51:263, 1986; Erlich, et al., European Patent Appln. 50,424; European Patent Appln. 84,796, European Patent Appln. 258,017, European Patent Appln. 237,362; Mullis, European Patent Appln. 201,184; Mullis, et al., U.S. Pat. No. 4,683,202; Erlich, U.S. Pat. No. 4,582,788; and Saiki, et al., U.S. Pat. No. 4,683,194). A number of PCR amplification methods are known to those of skill in the art and are used to identify nucleic acid sequences adjacent to a known sequence. For example, inverse PCR (IPCR) methods, which are used to amplify unknown DNA sequences adjacent to a core region of known sequence have been described. Other methods are also available such as capture PCR (Lagerstrom M., et al., PCR Methods Applic. 1:111, 1991), and walking PCR (Parker, et al., Nucleic Acids Res 19:3055, 1991). A number of manufacturers have also developed kits based on modifications of these methods for the purposes of identifying sequences of interest. Technical advances including improvements in primer and adaptor design, improvements in the polymerase enzyme, and thermocycler capabilities have facilitated quicker, efficient methods for isolating sequences of interest.

TABLE 5a

Primer sequences for isolation of Arabidopsis Actin and EF1α promoter sequences

At. Actin 2 forward: TTTTTTTTGATATCAAGCTTCAACTATTTTTATGTATGC SEQ ID NO: 1
At. Actin 2 reverse: GCCTCAGCCATGGTGAGTCTGCTGCAAACACACAAAAAGA GTTCAAT SEQ ID NO: 2
At. Actin 8 forward: TTTTTTTTGATATCAAGCTTCCATTTTTCTTTTGCATAATTC SEQ ID NO: 3
At. Actin 8 reverse: GCATCGGCCATGGTGAGTCTTCTGCAATCAAAAACATAAA GATCTGA SEQ ID NO: 4
At. Actin 11 forward: TTTTTTTTTAAGCTTGATATCACAACCAAATGTCAAATGG SEQ ID NO: 5
At. Actin 11 reverse: CCATCTGCCATGGTCTATATCCTGTC SEQ ID NO: 6
At. EF1α forward: TTTTTTTTTAAGCTTGATATCGGAAGTTTCTCTCTTG SEQ ID NO: 7
At. EF1α reverse: CTTTTCCCATGGTAGATCTCTGGTCAACAAATC SEQ ID NO: 8
At. Actin 1a forward: CCCAAGCTTAAATGACATCAGATACACGC SEQ ID NO: 13
At. Actin 1b forward: CATAAGCTTAGAGGTCCAAATTCA SEQ ID NO: 14
At. Actin 1 reverse: CCATCAGCCATGGTCTTCTACCTTTATGCAAA SEQ ID NO: 15
At. Actin 3 forward: CCAAGCTTACCACACTCAGATGCATAAACAAACACA SEQ ID NO: 16
At. Actin 3 reverse: CATCAGCCATGGTCTACTCTCTGCAAAAACA SEQ ID NO: 17
At. Actin 7 forward: GCAAAGCTTACTAGTCAACAATTGGCC SEQ ID NO: 18
At. Actin 7 reverse: GATCGGCCATGGTTCACTAAAAAAAAAG SEQ ID NO: 19
At. Actin 12 forward: GGAAGCTTGCGGCCGCTTTCTACTCTACATGTTTCT SEQ ID NO: 20
At. Actin 12 reverse: GACTAGCCGCCATGGTTCAATCTCTAGCTGA SEQ ID NO: 21

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The leaves of young plants of *Arabidopsis thaliana* (1 g) were homogenized in 9 ml of CTAB buffer (Saghai-Maroof et al. 1984, PNAS 81:8014–8018). The CTAB buffer contained 100 mM TrisHCl, pH 7.8, 700 mM NaCl, 50 mM EDTA, 1% CTAB (alkytrimethyhyl-ammoniumbromide) and 140 mM 2-mercaptoethanol. After 90 minutes incubation in 65 C., 4.5 ml of chloroform:isoamyl alcohol (24:1) was added and samples were mixed for 10 minutes. Aqueous layer was separated by centrifugation for 10 minutes at 1500 g and was re-extracted with chloroform:isoamyl alcohol. After second centrifugation, aqueous layer was transferred to a tube containing 50 μl 10 mg/ml RNase A (DNase free) and incubated in room temperature for 30 minutes to remove RNA. DNA was precipitated with 6 ml of isopropanol and re-suspended in 1 ml of 10 mM TrisHCl buffer pH 8.5. DNA solution was extracted once with equal volume of phenol and once with an equal volume of chloroform: isoamylalcohol. After centrifugation, 1/10 volume of sodium acetate (3M, pH 5.2) was added to aqueous layer, followed by 2.5 volume of ethanol. The DNA was hooked, washed in 70% ethanol, then air dried and re-suspended in 0.2 ml of 10 mM TrisHCl buffer.

Arabidopsis genomic DNA (100 ng) was used in 50 μl PCR reactions. Reactions containing the primers shown in Table 5. contained 10 μM reverse and forward primer solutions, 200 nM dNTPs and PCR buffer with magnesium and DNA polymerase mix from Expand™ High Fidelity PCR System (Roche Molecular Biochemicals). After initial 2 minute denaturation at 94° C. reactions were cycled 0.5 min at 94° C., 0.5 min at 55° C. and 1.5 minute at 72° C. for 35 times. PCR products were analyzed by electrophoresis on 1% agarose gel. Gel isolated DNA fragments representing Actin 1a, Actin 1b, Actin 7, and Actin 12 sequences were phosphorylated with T4 DNA kinase and ligated to dephosphorylated and Sma I cut pUC19 cloning construct. White colonies were screened for the presence of appropriate inserts and sequenced with M13 forward and reverse primers to confirm the presence of actin promoters. Selected clones were designated as pMON54941 (P-AtAct1a), pMON54942 (P-AtAct1b), pMON54943 (P-AtAct7) and pMON54944 (P-AtAct12). Subsequently, the Actin promoters DNA fragments were released by Hind III and NcoI digest of the pUC19 constructs containing the insert sequences, the DNA fragments were gel isolated and ligated to pMON26165 that had been digested with the same restriction enzymes. A PCR product for the Actin 3 promoter (P-AtAct3) was digested with Hind III and Nco I and cloned directly into pMON26165 to form pMON54951. pMON26165 contains the GUS/nos terminator gene segment. Ligation with the promoter segments allows for assay of each promoter for functional activity by expression of the β-glucuronidase enzyme in plant cells. The plant cells can be isolated, for example, tobacco leaf protoplasts, or the plant cells may be contained in a plant tissue or organ, such as, leaf, root, cotyledon, hypocotyl, embryo, flower, or storage organ. The expression level of GUS driven by these promoters is assayed in soybean hypocotyl in comparison with GUS driven by P-e35S promoter (Table 6). Plasmid DNA/gold particles was bombarded to soybean hypocotyls then after 48 hours the GUS activity was assayed histochemically. All of the Actin promoters tested in this assay show functional activity in the hypocotyl tissue demonstrating their utility for expression transgenes in heterologous crop plant species.

The constructs containing aroA:CP4 EPSPS driven by the Arabidopsis Actin 1a, (pMONT54952), Actin 1b (pMON54953), Actin 3 (pMON54954), Actin 7 (pMON54955) and Actin 12 (pMON54956) promoters of the present invention were prepared in Agrobacterium binary plant transformation constructs for stable expression of the glyphosate resistant EPSPS in crop plants. These constructs are transformed into soybean and cotton cells, the cells are selected and regenerated into plants on glyphosate containing tissue culture media and then assayed for expression of the aroA:CP4 protein and for tolerance to glyphosate application. Plants demonstrating commercially acceptable glyphosate tolerance are further developed by conventional breeding methods to transfer the glyphosate tolerance trait into germplasm adapted for cultivation.

TABLE 6

Activity of different Arabidopsis actin promoters in transient assay as compare to P-e35S.

Construct	GUS Activity
Pe35S/GUS	+++
P-AtAct1a/GUS	++
P-AtAct1b/GUS	++
P-AtAct3/GUS	++
P-AtAct7/GUS	++
P-AtAct12/GUS	+

Example 14

Cotton yield is correlated with the number of squares set during the first four to five weeks of squaring. The retention of these squares to mature bolls and their contribution to the harvest of the cotton lint is a key component of yield. When determining the efficacy of transgene constructs for conferring herbicide tolerance in cotton, the amount of boll retention is a measure of efficacy and is a desirable trait. Transgenic cotton plants containing promoters of the present invention (Table 7) were assayed in greenhouse conditions for boll retention. The promoters directed expression of the aroA:CP4 coding sequence for glyphosate tolerant phenotype. The plants were transformed by an Agrobacterium-mediated method or by a particle gun method. The particle gun constructs contained an additional GUS containing expression cassette useful for histochemical localization of β-glucuronidase activity from the promoters of the present invention. Transgenic plants were regenerated on glyphosate containing media and plants rooted on a rooting media. The rooted plantlets were potted in soil and transferred to a growth chamber for a hardening off period. The seed from these plant lines were collected and planted. Fifteen plants from each line were sprayed with glyphosate at 48 ounces/acre at the 4 leaf stage. At least 8 surviving plants from each line were sprayed again at the 8 leaf stage with glyphosate at 48 ounces/acre. At maturity, the number of first position bolls for the first five bolls was counted. Those lines that had 3 or more of the first position bolls retained after the glyphosate spray (plant map≧3) were advanced for further study. Table 7 illustrates the data produced from this study. The number of lines mapped indicates the number of lines surviving the first glyphosate spray application. The commercial standard is Line 1445 (pMON17136) that contains the P-FMV promoter driving expression of the CTP2-aroA:CP4 gene/E9 3', this line retains less than 1 of the 5 first bolls. The constructs, pCGN8099, pCGN9153, pCGN8088, pCGN8068 provided sufficient reproductive glyphosate tolerance in cotton such that 14–35% of the lines tested from these constructs were advanced for further agronomic trials.

TABLE 7

Greenhouse cotton boll retention study

Construct	Promoters	# lines Mapped	Plant Map ≧ 3	% ≧3
pCGN8099	FMV:EF1a + e35S:Act8	104	36	34.6%
pCGN9153	EF1α + FMV	36	12	33.3%
pCGN9165	EF1α +35S/GUS	3	1	33.3%
pCGN9152	EF1a	7	0	0.0%
pCGN8088	Act8 + FMV	43	6	14.0%
pCGN8086	Act8	7	0	0.0%
pCGN8068	Act2 + FMV	37	7	18.9%
pCGN8067	Act2	37	0	0.0%
pCGN8084	Act2 + FMV + 35S/GUS	5	0	0.0%
pCGN8085	Act2 + FMV/GUS	1	0	0.0%
pCGN9164	Act11 +35S/GUS	21	1	4.8%
pMON45325	Act11 + FMV	43	0	0.0%
pCGN8096	FMV:Act11 + e35S:Act2	14	0	0.0%
pCGN9154	FMV:Act11 + e355:Act2	16	1	6.3%
Line 1445	FMV		<1.0	

Example 15

Cotton yield is correlated with the number of squares set during the first four to five weeks of squaring. The retention of these squares to mature bolls and their contribution to the harvest of the cotton lint is a key component of yield. When determining the efficacy of transgene constructs for conferring herbicide tolerance in cotton, the amount of boll retention is a measure of efficacy and is a desirable trait. Transgenic cotton plants containing promoters of the present invention were assayed in field conditions at two locations for boll retention. The transgenic cotton lines 502-254-2 (pCGN8068), 701-178-2 (pCGN8068), 53-2 (pCGN8088), 178-1 (pCGN9153), and 60-1 (pCGN9153) were compared to 1445 (glyphosate tolerance line) and PM1218BR (Paymaster 1218 parent) that contain the construct pMON17136 (P-FMV/CTP2-aroA:CP4/E93'), a wild type non-transgenic line, Coker 130 was included. The field design is a randomized complete block design consisting of 2 rows×20–30 feet×3 replications. Glyphosate is applied as Roundup Ultra™ formulation at rates of 1.12 lb ai/A=48 oz product and 1.5 lb ai/A=64 oz product at the 8 leaf stage of cotton plant development. All of the cotton plots are managed aggressively for weed and insect pest control, as well as other agronomic inputs such as planting time, fertilization, irrigation, PGR usage and defoliation. The percent boll retention is determined by mapping the location of each of the retained bolls by random selection of ten plants from the middle of the two center rows (five from each row) of each plot to map. The first mapping should be done 4 weeks after first flower (mid-season map), a second mapping should be done at harvest. The data collected includes the number of first position bolls on the bottom five flowering nodes that are counted as an indication of the reproductive tolerance of the transgenic cotton lines to glyphosate. Table 8 illustrates the advantage that promoters of the present invention have conferred to transgenic cotton plants for boll retention. This enhanced reproductive tolerance has resulted in increased lint yield (Table 9) and increased seed yield (Table 10) as well.

TABLE 8

Boll retention at mid-season plant map of bottom 5 first position bolls

	Location 1			Location 2		
	Untreated	48 oz/A	64 oz/A	Un-treated	48 oz/A	64 oz/A
(17136) 1445	68	67	53	81	63	62
(8068) 502-254-2	87	72	64	77	80	69
(8068) 701-178-2	85	77	60	84	86	76
(8088) 53-2	89	81	80	79	76	73
(9153) 178-1	77	83	73	85	71	79
(9153) 60-1	80	89	81	77	82	87
PM1218BR	92	56	63			

TABLE 9

Lint Yield (lbs/Acre) and percent yield (Location 1)

Cultivar	Untreated	48 oz/A	48 oz/A %	64 oz/A	64 oz/A %
8068-502-254-2-4	1103	960	87.0%	858	77.8%
8068-701-178-2-2	1326	1219	91.9%	1177	88.8%
9153-60-1-1	1177	1206	102.5%	1171	99.5%
9153-178-1-1	1112	769	69.2%	750	67.4%
8088-53-2-11	1283	1071	83.5%	1097	85.5%
1445	1018	563	55.3%	490	48.1%
C130	1200	0	0.0%	0	0.0%
PM 1218 BR	1092	826	75.6%	713	65.3%

TABLE 10

Seed Cotton Yield (lbs/Acre) and percent yield (Location 1)

Cultivar	Untreated	48 oz/A	48 oz/A %	64 oz/A	64 oz/A %
8068-502-254-2-4	3357	2923	87.1%	2646	78.8%
8068-701-178-2-2	3720	3521	94.7%	3328	89.5%
9153-60-1-1	3294	3413	103.6%	3316	100.7%
9153-178-1-1	3468	2355	67.9%	2218	64.0%
8088-53-2-11	3404	2950	86.7%	2968	87.2%
1445	2835	1624	57.3%	1372	48.4%
C130	3272	0	0.0%	0	0.0%
PM 1218 B/RR	3036	2192	72.2%	1885	62.1%

Example 16

The efficacy of the hybrid promoter P-FMV-AtEF1α driving expression of the CTP2-aroA:CP4 coding sequence (FIG. 13, pMON52059) and P-FMV/CTP2-aroA:CP4/E93' (pMON15737) was compared in transgenic *Arabidopsis thaliana*. The transgenic *Arabidopsis thaliana* plants were produced by the vacuum infiltration (Bechtold et al., C R Acad Paris Life Sci 316: 1194–1199) seeds were potted in soil in trays in a growth chamber adjusted for 24° C., 16 hour light (120 μE $m^{-2}s^{-1}$) cycle to permit normal growth and development of the plants. The pMON52059 V1 event glyphosate tolerant transgenic Arabidopsis plants were selected by spray application of glyphosate herbicide at a rate of 24 ounces/acre, the surviving plants were transplanted into individual pots. Eight pMON52059 V1 plants and eight pMON15737 homozygous plants were sprayed a second time corresponding to the observation of bolting, approximately 16 days after the at a rate of 24 ounces/acre. The second spray will determine the efficacy of the two constructs for conferring reproductive tolerance. The plants were observed for vegetative effects of glyphosate application. All plants had complete vegetative tolerance and no

abnormal flowers were observed. However, abortion of siliques occurred indicated that seed had not been set in the aborted siliques. The total number of siliques produced by each plant and the siliques that contained seeds (fertile siliques) were counted and tabulated. The results are shown in Table 9 and indicate that the hybrid promoter construct pMON52059 demonstrated a greater than 10 fold improvement in fertile siliques, 89% compared to pMON15737 at 8%. The number of fertile fruiting structures is related to the amount of seed that can be produced, this is especially important in crops whose yield is associated with seed numbers. Crops such as cotton, soybean, canola, wheat, and corn are crops where reproductive tolerance to glyphosate is essential for good yield.

TABLE 11

Comparison of the hybrid promoter P-FMV-EF1α (pMON52059) and P-FMV (pMON15737) in conferring reproductive tolerance to glyphosate in Arabidopsis plants.

pMON52059				pMON15737			
Plant Number	Fertile Siliques	Total Siliques	Percent Fertility	Plant Number	Fertile Siliques	Total Siliques	Percent Fertility
8819	39	50	78.0%	1	74	540	13.7%
8820	626	691	90.6%	2	23	600	3.8%
8821	507	561	90.4%	3	1	470	0.2%
8822	0	69	0.0%	4	20	646	3.1%
8823	512	534	95.9%	5	43	717	6.0%
8827	326	354	92.1%	6	22	651	3.4%
8833	432	461	93.7%	7	178	868	20.5%
8838	323	374	86.4%	8	40	520	7.7%
Total	2765	3094	89.4%	Total	401	5012	8.0%

Example 17

Sunflower (*Helianthus annuus* L.) is a crop of agronomic importance for oil and food. The constructs pMON45325 (FIG. 2), pMON45332 (FIG. 4), and pMON45331 (FIG. 3) of the present invention were transformed into sunflower. Agrobacterium-mediated transformation of sunflower has been reported (Schrarmneijer et al., *Plant Cell Reports*, 9: 55–60, 1990; EP 0 486 234). Methods known by those skilled in the art of plant transformation with transgene expression constructs can include hypocotyls, apical meristems, protoplasm, and other sunflower tissues. Transgenic sunflower lines SFB250-27 contains pMON20999 (P-FMV/CTP2-aroA:CP4/E93') expression cassette; SFB288-01, SFB295-09 contain pMON45325 (P-eFMV/CTP2-aroA:CP4/E93'::P-AtAct11+intron/CTP2-aroA:CP4/E93'); SFB289-01 contain pMON45332 (P-AtEF1α+intron/CTP2-aroA:CP4/E93'::P-eFMV/CTP2-aroA:CP4/E93'); SFB303-08, SFB303-09, SFB303-11, and HA300B contain pMON45331 (P-AtEF1α+intron/CTP2-aroA:CP4/E9). These lines are tested for glyphosate tolerance and are shown in Table 12.

The reproductive tolerance to glyphosate in sunflower can be measured as a function of the precent of normal heads, percent normal head size and the pollen production. These plants are sprayed with Glyphosate at V-4 and V-8 leaf stages at 0, 32 oz/acre or 64 ounces/acre rate. The sunflower plants are assessed for vegetative tolerance to glyphosate. Vegetative tolerance is achieved at 32 and 64 oz/acre levels of glyphosate spray at both V4 and V8 stages of plant development.

Vegetative glyphosate tolerant transgenic sunflower lines are scored for number of heads; precent normal heads, percent normal head size, and percent normal pollen shed. These traits are scored in a field test at one location. The tabulation of the head scores and pollen production is shown in Table 12. Lines selected from the constructs of the present invention show greater percent of normal heads, generally greater percent normal head size and better pollen shed.

TABLE 12

Sunflower glyphosate resistance scores

Line #	# heads	% normal heads	% normal head size	% pollen shed
SFB250-27	28	29	75	36
SFB288-01	11	36	73	73
SFB295-09	28	57	64	68
SFB289-01	13	38	92	38
SFB303-08	25	68	92	64
SFB303-09	43	81	88	88
SFB305-11	45	71	84	100
HA300B	30	100	97	97
non-trans segregant	0	0	0	0

Example 18

Cis acting regulatory elements necessary for proper promoter regulation can be identified by a number of means. In one method, deletion analysis is carried out to remove regions of the promoter and the resulting promoter fragments are assayed for promoter activity. DNA fragments are considered necessary for promoter regulation if the activity of the truncated promoter is altered compared to the original promoter fragment. Through this deletion analysis, small regions of DNA can be identified which are necessary for positive or negative regulation of transcription. Promoter sequence motifs can also be identified and novel promoters engineered to contain these cis elements for modulating expression of operably linked transcribable sequences. See for example U.S. Pat. No. 5,223,419, herein incorporated by reference in its entirety, U.S. Pat. No. 4,990,607 herein incorporated by reference in its entirety, and U.S. Pat. No. 5,097,025 herein incorporated by reference in its entirety.

An alternative approach is to look for similar sequences between promoters with similar expression profiles. Promoters with overlapping patterns of activity can have common regulatory mechanisms. Several computer programs can be used to identify conserved, sequence motifs between promoters, including but not limited to MEME, SIGNAL SCAN, or GENE SCAN These motifs can represent binding sites for transcriptions factors which act to regulate the promoters. Once the sequence motifs are identified, their function can be assayed. For example, the motif sequences can be deleted from the promoter to determine if the motif is necessary for proper promoter function. Alternatively, the motif can be added to a minimal promoter to test whether it is sufficient to activate transcription. Suspected negative regulatory elements can be tested for sufficiency by adding to an active promoter and testing for a reduction in promoter activity. Some cis acting regulatory elements may require other elements to function. Therefore, multiple elements can be tested in various combinations by any number of methods known to those of skill in the art.

Once functional promoter elements have been identified, promoter elements can be modified at the nucleotide level to affect protein binding. The modifications can cause either higher or lower affinity binding which would affect the level of transcription from that promoter.

Promoter elements can act additively or synergistically to affect promoter activity. In this regard, promoter elements from different 5' regulatory regions can be placed in tandem to obtain a promoter with a different spectrum of activity or different expression profile. Accordingly, combinations of promoter elements from heterologous sources or duplication of similar elements or the same element can confer a higher level of expression of operably linked transcribable sequences. For example, a promoter element can be multimerized to increase levels of expression specifically in the pattern affected by that promoter element.

The technical methods needed for constructing expression constructs containing the novel engineered 5' regulatory elements are known to those of skill in the art. The engineered promoters are tested in expression constructs and tested transiently by operably linking the novel promoters to a suitable reporter gene such as GUS and testing in a transient plant assay. The novel promoters are operably linked to one or more genes of interest and incorporated into a plant transformation construct along with one or more additional regulatory elements and transformed into a target plant of interest by a suitable DNA delivery system. The stably transformed plants and subsequent progeny are evaluated by any number of molecular, immunodiagnostic, biochemical, phenotypic, or field methods suitable for assessing the desired characteristic(s).

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

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atttaaatgg gtaatcatat atttgtgtcc agtggattga acaaatatgc tcggcccatg 600

cggaagtaat gccaattttg ggtgagtaaa gcccatgcga aattttcaca taagaaatgc 660

atgctttttg ttttcaacga catgagttgc atgctttta tcattgctta tatagttgca 720

agtttgcaac tccttgatat ttttttatg tagacactac taccaccaaa aacttttggt 780

ctgcttattc ttgtttacta tgtaaaaaaa ataaatgaat tgtttattta ctccgatttg 840

atggagtctg gtttatgagg ttttatagcc tttacagaaa attgatagtt acaaaaatat 900

ttttcaaaaa taaaagggta aaaccgtcat ttcaagttgt tattgttttg ggggactgga 960

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tttgaaatga aatatagaac cggaaaacaa ggtgagccga agtcgaagcc tttggacccg 1020

tttttatatt tactcctccc attcccttct ccttcaatcc ttccttcctc ctcctccctt 1080

cttcttcttc ccctctttca ttttccagcc actacaaact tttctatctc tacttttttt 1140

cctctcgatt tcaggtactt tttgagaccc tttgttgtga ttttcgaaca cacaccccaa 1200

ttacgtttga tttttgatcc cgcatcgatt tcaattcatc cgtttctgag tttcttttgg 1260

atctgggtgt cttgagctaa tcttttcgat ctgttgttta tcgattttac tcatgcgtat 1320

gttcattaca ccatttgtta tttgtttaat caaccaaaag actcatgttt ttcaaatgtc 1380

tttaatataa tttttctgat tgaattttat aatatttaca tgattctgga tccagaatat 1440

ccttcttctt cttccatttt gtcctgtatt gatttgtctt tgaaaaagga ttgttctttg 1500

tatctgtatt ggtgaaaaag gattgttatt tgttgataaa aatttgatct ttaaacaatg 1560

tttggttttg cataaagg 1578

<210> SEQ ID NO 23
<211> LENGTH: 1468
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1468)
<223> OTHER INFORMATION: Act1b promoter polynucleotide sequence and intron

<400> SEQUENCE: 23

ttagaggtcc aaattcaaaa aaatgtcgta ttgaatcatt ccattactaa attggttcaa 60

tgtcagattt aaacagccta gggataattt agtgagatat gagattctac tttcaacata 120

tactaatcct aaatctctag caacttttta tataagctat aaatatcatg aaaatgtatt 180

ttaatcgttt cataatttat gcagtcacac taatggaaaa aaggccaatt attattattt 240

tcttcagact ataaatgaaa acataaatta aaatgcagat tagtttaaaa ttttaataag 300

taagtaaaat gcttatagcc ttatacaaaa tcatatttgg aagtttctaa cattgttgca 360

atttgttatc acaaatcaca gtaatatttg tatactaatt agtaattaca actatacaca 420

aatttaaatg ggtaatcata tatttgtgtc cagtggattg aacaaatatg ctcggcccat 480

gcggaagtaa tgccaatttt gggtgagtaa agcccatgcg aaattttcac ataagaaatg 540

catgcttttt gttttcaacg acatgagttg catgcttttt atcattgctt atatagttgc 600

aagtttgcaa ctccttgata ttttttttat gtagacacta ctaccaccaa aaacttttgg 660

tctgcttatt cttgtttact atgtaaaaaa aataaatgaa ttgtttattt actccgattt 720

gatggagtct ggtttatgag gttttatagc ctttacagaa aattgatagt tacaaaaata 780

tttttcaaaa ataaaagggt aaaaccgtca tttcaagttg ttattgtttt gggggactgg 840

atttgaaatg aaatatagaa ccggaaaaca aggtgagccg aagtcgaagc ctttggaccc 900

gtttttatat ttactcctcc cattcccttc tccttcaatc cttccttcct cctcctccct 960

tcttcttctt cccctctttc attttccagc cactacaaac ttttctatct ctacttttt 1020

tcctctcgat ttcaggtact ttttgagacc ctttgttgtg attttcgaac acacacccca 1080

attacgtttg atttttgatc ccgcatcgat ttcaattcat ccgtttctga gtttcttttg 1140

gatctgggtg tcttgagcta atcttttcga tctgttgttt atcgatttta ctcatgcgta 1200

tgttcattac accatttgtt atttgtttaa tcaaccaaaa gactcatgtt tttcaaatgt 1260

ctttaatata atttttctga ttgaatttta taatatttac atgattctgg atccagaata 1320

-continued

tccttcttct tcttccattt tgtcctgtat tgatttgtct ttgaaaaagg attgttcttt 1380

gtatctgtat tggtgaaaaa ggattgttat ttgttgataa aaatttgatc tttaaacaat 1440

gtttggtttt gcataaaggt agaagacc 1468

<210> SEQ ID NO 24
<211> LENGTH: 1642
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1642)
<223> OTHER INFORMATION: Act3 promoter polynucleotide sequence and intron

<400> SEQUENCE: 24

tcaagcttac cacactcaga tgcataaaca aacacagcaa gaagattgcc acaaaaatca 60

taacgaaata atcaagagat agctatcaaa tcgccaccgg cgaatcatgt catactcagt 120

atcagaaaca gatatgatag ctcaaaatat ggattaataa tgttactaaa cacatggaca 180

ataatgcatc aatattgaaa gaaagaaaat ggtttagcag aagcaaaatg gtttagaaag 240

taatgaacta cacattcaca aaggtgaaga attcgtcaag cctacaataa caaatgtcta 300

tactttatga gcccacaaag agatacatca cactatctga acgaaactaa agcaacctaa 360

catagtctag aaactactaa aatgaatgtt tcaaaacaat tttaacagaa ggcaaaagtg 420

aaacaacata ctcctttgcg agaacgagga cgaggagcta attcacgtct ggtaacaaca 480

tgtcccttgt tcaacccaac gaacaaaccg gtcttcactt gtggagttgt catcttctgt 540

aaatttcata gacaacaaac aaacaaaact ttctattcaa tacaaaatca aattttacaa 600

gagacggatt cagagataat aaagagatga agagagttaa atcaaaaggg attgatagaa 660

gatacctaat caatggatcg agctcctccg gtggttcaga caaaagaagg acgccgactg 720

aaaattacat ttttgtatat ataccagaga gactcaagaa aaaaccctag tccagtttgg 780

gcttttattg ggccttataa attttgggtc agttttgaca aagtaaatac aaggctatag 840

ctgctttgct aacgtgatta attatttacc atttaccaaa agccttaacc gaggccgagc 900

gagaaaaaaa aacaaaaaaa aggtagaggg caagaacgtc atttccacaa ggaattgaat 960

cggaaaacga ggtgtgccga attcgaagcc tttggacccg tttttatact tttttacttg 1020

ccattcgttt ttttttgttc attggcctca tttgattact tgtttctttg atttctcctt 1080

ccatagaacc gaattgtttt cagtctgaga tttctcctgc cgagagaacg attttaatct 1140

attttcctcg gtaatgttat agcctaattt gtgtttttt cttttcctg atccggatat 1200

cgttattctg attgacaatt gtcagtttca tcttctattc tgtgaaattt tgatttttt 1260

ccgatctgtg atttcgtcat tgtatcagcg tgcttatatg cgtttgaggc gtaaatgagt 1320

gtgtacctca tttatcattt gctatgtttt ttttttaac agagatcttc agctgtaata 1380

ttataataga ttgaattgat aacgtgattc tggatctgga atatatatat gtcacattct 1440

tcttaggatt tgattttgtc tctctttgga tattaatatt cttcactccc ttgaaaatga 1500

atctgtttat tataatgttt agatatattc cttaccggca tttgttttag cataaatatg 1560

aaacatagca ttgactgatt tgtcttttta ttattcttgt ttttttgcca aattggtctc 1620

atgtttttgc agagagtaga cc 1642

<210> SEQ ID NO 25
<211> LENGTH: 1241
<212> TYPE: DNA

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<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1241)
<223> OTHER INFORMATION: n= a or g or c or t/u
Act7 promoter polynucleotide sequence and intron

<400> SEQUENCE: 25

actagtcaac aattggccaa tctttngttc taaattgcta ataaacgacc atttccgtca 60

attctccttg gttgcaacag tctacccgtc aaatgtttac taatttataa gtgtgaagtt 120

tgaattatga aaaacgaaat cgtattaaaa attcacaaga ataaacaact ccatagattt 180

tcaaaaaaac agtcacgaga aaaaaaccac agaccgtttg tctgctcttc tagtttttat 240

tatttttcta ttaatagttt tttgttattt cgagaataaa atttgaacga tgtccgaacc 300

acaaaagccg agccgataaa tcctaagccg agcctaactt tagccgtaac catcagtcac 360

ggctcccggg ctaattcatt tgaaccgaat cataatcaac ggtttagatc aaactcaaaa 420

caatctaacg gcaacataga cgcgtcggtg agctaaaaag agtgtgaaag ccaggtcacc 480

atagcattgt ctctcccaga ttttttattt gggaaataat agaagaaata gaaaaaaata 540

aaagagtgag aaaaatcgta gagctatata ttcgcacatg tactcgtttc gctttcctta 600

gtgttagctg ctgccgctgt tgtttctcct ccatttctct atctttctct ctcgctgctt 660

ctcgaatctt ctgtatcatc ttcttcttct tcaaggtgag tctctagatc cgttcgcttg 720

attttgctgc tcgttagtcg ttattgttga ttctctatgc cgatttcgct agatctgttt 780

agcatgcgtt gtggttttat gagaaaatct ttgttttggg ggttgcttgt tatgtgattc 840

gatccgtgct tgttggatcg atctgagcta attcttaagg tttatgtgtt agatctatgg 900

agtttgagga ttcttctcgc ttctgtcgat ctctcgctgt tatttttgtt tttttcagtg 960

aagtgaagtt gtttagttcg aaatgacttc gtgtatgctc gattgatctg gttttaatct 1020

tcgatctgtt aggtgttgat gtttacaagt gaattctagt gttttctctt tgagatctgt 1080

gaagtttgaa cctagttttc tcaataatca acatatgaag cgatgtttga gtttcaataa 1140

acgctgctaa tcttcgaaac taagttgtga tctgattcgt gtttacttca tgagcttatc 1200

caattcattt cggtttcatt ttacttttttt tttagtgaac c 1241

<210> SEQ ID NO 26
<211> LENGTH: 1313
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1313)
<223> OTHER INFORMATION: polynucleotide sequence of Act12 promoter and intron

<400> SEQUENCE: 26

tttctactct acatgtttct tgttattagg taaagtatta ggctcttttt ttaaaaaaaa 60

tgcttaatcc tctgggtacc tcgaaaaggg aataatactc tagttagata agtgcagcga 120

tcaacatgac aaaatgaatg aatgtttgct ttaattggtg gctaaaagct aaatacacag 180

aaaagtcaaa attcaatctc aaaatcaacc cctctgtctc caatgtccct aatctatacc 240

aaaatgtcaa tttattttct tgatcatata ttccactaat taaaaataaa tccttctcta 300

atgaaatttg tcaaggcctt ggaagcctag ttttaaatat taaatggaaa ctatttcttc 360

aacaatcaca ctgttattta gtattgttgt atgttgttca ctactttctt catttgtttt 420

gtaagaaact ataataagca aaaacacata ataaagtctc atgtcaaata atgaatctta 480

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tgcacatgct tgattatttt acttgcacat atccctatca tcattatcac atttgtcaat 540

taccgttatc atcattactc tcattcttcc cagaactttt tcagcaattt ccatacctca 600

cccactaaga tcttttaccc tttttcttaa ttatagtttg gatagcactc ttttacatag 660

cactgaaatt tcggttgaac acataaatta ctagaaacta gaaggaaatg ttactgaaat 720

ttcactgatt gtctaaaatt gaataatcta aagaaaatgg ccttttaacc tttttcttag 780

gcccaaatgg gctcattacc actcatgctt gttcggtgac ccgattcttc cggtaaaaca 840

gagcctaaac cgtattttca ggttaggctg gtgttttctt aattctccaa cctaaaaata 900

gatggacacg tgtctataga ggctgagata ttggtctcaa tgaagaaaac taacggctca 960

gacccgtgta tgaacgatat taagggccaa agttgcttct gttttccaga aatttttgaa 1020

acccaatttc agggcacgat tccacaacct ctttcttttc ttctagatct acgtaaattc 1080

atcaggtaca tgttattttt tttgtttatt tgatgtcaaa attttgatca caaggaggca 1140

aaaccaatat aaatgtaacg ctaatgcgtt tgattatggt atacgtaacg aattagattt 1200

aatggttaca ttttattgtt ttagatttag ttatgagatt ggcattaatt attggtgttt 1260

cctttgaatt tgctatgttt cttatgttga tgtaatcagc tagagattga acc 1313

<210> SEQ ID NO 27
<211> LENGTH: 1946
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1946)
<223> OTHER INFORMATION: chimeric promoter fusion FMV and Act11
polynucleotides + Act11 intro

<400> SEQUENCE: 27

aattctcagt ccaaagcctc aacaaggtca gggtacagag tctccaaacc attagccaaa 60

agctacagga gatcaatgaa gaatcttcaa tcaaagtaaa ctactgttcc agcacatgca 120

tcatggtcag taagtttcag aaaaagacat ccaccgaaga cttaaagtta gtgggcatct 180

ttgaaagtaa tcttgtcaac atcgagcagc tggcttgtgg ggaccagaca aaaaaggaat 240

ggtgcagaat tgttaggcgc acctaccaaa agcatctttg cctttattgc aaagataaag 300

cagattcctc tagtacaagt ggggaacaaa ataacgtgga aaagagctgt cctgacagcc 360

cactcactaa tgcgtatgac gaacgcagtg acgaccacaa aagaattagc ttgagctcag 420

gatttagcag cattccagat tgggttcaat caacaaggta cgagccatat cactttattc 480

aaattggtat cgccaaaacc aagaaggaac tcccatcctc aaaggtttgt aaggaagaat 540

tcgatatccc cgcggccgcg ttatcacaac caaatgtcaa atggaatgca tcagagacca 600

aacctgtaag agtccacaaa acaattcaaa gaaagaatat caacaattca gagattcaat 660

cctaaaacaa aaagagaact gaaaccaaat cgtacctaca cgaccagtga agataccaat 720

agagagctct gttgtagaat acaacacatt aagcgcaatt agcagaaaca gtctcttcat 780

ctgccgattt ccacttgtca ctactccaaa aacctcccaa accatttcca aaacagacac 840

ttttgccatg tctacatctt tcccttcccc gaaaaacaca tcatttccat caacggagta 900

aatatccggc ggcatatcga tgctcgagac cgtcctatcg agaaaaggct tagccgcttc 960

cgtgaccgcc ggcgttcgtg gaccgtgaga ttgctgaaac gagcgagaat aagcaagcct 1020

ccgatcatta gcagcatatc cgacatcgct gctccgatca tcagggagct cgttatcgcc 1080

tcgaggatta aaggaaatgg atctctccat tttcttcttt gatcttaaag ttccaacttc 1140

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ggcaaatact aaaatcaaca gtcagtcgta caaagaaact ctgcttatac agtaaagtca 1200

atgggccact gttctaagcc catatataat tttagaagcc catagaatac aaaagagtca 1260

agaagcattg accgcacaag aaaaaaacaa ttgttaaaaa gggttggtta gtgtgtatgt 1320

atatatgaaa tgcaacaaac attatacagc ccattaaata tggttgttat aggtagatgt 1380

ccccattaag gaactttatc cagcccatta aattacttta cagagtaaaa gagagagaga 1440

agatttacag ttacgttacc aaattttcga aatgatttaa ttagtaataa ataaataatt 1500

aaatgtcagt tactctcttt agaaagctaa ataagacagc tgtttccacc aacaacgtga 1560

ctggtcgtgg ggtcctcctt cgttcaaagt gatattcaga aatcaacggc tgagatcttc 1620

tccatcaata tttattacgg gcctattcct tccttttta aacttcaatt ctccggctca 1680

cattctcttc ttcattcgct ccgtttctct ctcaaaaact acacacccgt accacaccac 1740

caccctcctc gtttcctcag agatcccctc tctaacttct aaggtaatca catttccata 1800

acgttccatc gtcattgatt cttcattagt atgcgtttat gaagcttttt caatttaatt 1860

ctctttggta gatcttaaga ttcctctgtt tcttgcaaaa taaagggttc aattatgcta 1920

atatttttta tatcaatttt gacagg 1946

<210> SEQ ID NO 28
<211> LENGTH: 1695
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1695)
<223> OTHER INFORMATION: n= a or g or c or t/u, r = g or a, y = t/u or c
chimeric promoter fusion FMV and EF1 polynucleotides + EF1 intro

<400> SEQUENCE: 28

aattctcagt ccaaagcctc aacaaggtca gggtacagag tctccaaacc attagccaaa 60

agctacagga gatcaatgaa gaatcttcaa tcaaagtaaa ctactgttcc agcacatgca 120

tcatggtcag taagtttcag aaaaagacat ccaccgaaga cttaaagtta gtgggcatct 180

ttgaaagtaa tcttgtcaac atcgagcagc tggcttgtgg ggaccagaca aaaaaggaat 240

ggtgcagaat tgttaggcgc acctaccaaa agcatctttg cctttattgc aaagataaag 300

cagattcctc tagtacaagt ggggaacaaa ataacgtgga aaagagctgt cctgacagcc 360

cactcactaa tgcgtatgac gaacgcagtg acgaccacaa aagaattagc ttgagctcag 420

gatttagcag cattccagat tgggttcaat caacaaggta cgagccatat cactttattc 480

aaattggtat cgccaaaacc aagaaggaac tcccatcctc aaaggtttgt aaggaagaat 540

tcgatatcaa gcttgatatc ggaagtttct ctcttgaggg aggttgctcg tggaatggga 600

cacatatggt tgttataata aaccatttcc attgtcatga gattttgagg ttaatatata 660

ctttacttgt tcattatttt atttggtgtt tgaataaatg atataaatgg ctcttgataa 720

tctgcattca ttgagatatc aaatatttac tctagagaag agtgtcatat agattgatgg 780

tccacaatca atgaaatttt tgggagacga acatgtataa ccatttgctt gaataacctt 840

aattaaaagg tgtgattaaa tgatgtttgt aacatgtagt actaaacatt cataaaacac 900

aaccaaccca agaggtattg agtattcacg gctaaacagg ggcataatgg taatttaaag 960

aatgatatta ttttatgtta aaccctaaca ttggtttcgg attcaacgct ataaataaaa 1020

ccactctcgt tgctgattcc atttatcgtt cttattgacc ctagccgcta cacacttttc 1080

tgcgatatct ctgaggtaag cgttaacgta cccttaratc gttcytttte yttttcgtct 1140

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gctgatcgtt gctcatatta tttcgatgat tgttggattc gatgctcttt gttgattnat 1200

cgttctgaaa attctnatct gttgtttaga ttttatcgat tgttaatatc aacgtttcac 1260

tgcttctaaa cgataattta ttcatgaaac tattttccca ttctgatcga tcttgttttg 1320

agattttaat ttgttcgatt gattgttggt tggtggatct atatacgagt gaacttgttg 1380

atttgcgtat ttaagatgta tgtcgatttg aattgtgatt gggtaattct ggagtagcat 1440

aacaaatcca gtgttccctt tttctaaggg taattctcgg attgtttgct ttatatctct 1500

tgaaattgcc gatttgattg aatttagctc gcttagctca gatgatagag caccacaatt 1560

tttgtggtag aaatcggttt gactccgata gcggcttttt actatgattg ttttgtgtta 1620

aagatgattt tcataatggt tatatatgtc tactgttttt attgattcaa tatttgattg 1680

ttctttttttt tgcag 1695

<210> SEQ ID NO 29
<211> LENGTH: 1800
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1800)
<223> OTHER INFORMATION: y = t/u or c
chimeric promoter fusion CaMV and Act8 polynucleotides + Act8 in
tro

<400> SEQUENCE: 29

ggtccgatgt gagacttttc aacaaagggt aatatccgga aacctcctcg gattccattg 60

cccagctatc tgtcacttta ttgtgaagat agtggaaaag gaaggtggct cctacaaatg 120

ccatcattgc gataaaggaa aggccatcgt tgaagatgcc tctgccgaca gtggtcccaa 180

agatggaccc ccacccacga ggagcatcgt ggaaaaagaa gacgttccaa ccacgtcttc 240

aaagcaagtg gattgatgtg atggtccgat gtgagacttt tcaacaaagg gtaatatccg 300

gaaacctcct cggattccat tgcccagcta tctgtcactt tattgtgaag atagtggaaa 360

aggaaggtgg ctcctacaaa tgccatcatt gcgataaagg aaaggccatc gttgaagatg 420

cctctgccga cagtggtccc aaagatggac ccccacccac gaggagcatc gtggaaaaag 480

aagacgttcc aaccacgtct tcaaagcaag tggattgatg tgatatcaag cttccatttt 540

tcttttgcat aattcatgtt tatttttta ttttttcat cttgcataat tcatgtttaa 600

aaggatatat acatgggtct actacattct cctgacatta cgttttatgt gtttgtcttc 660

tgaaaataat catcaaaata tttcaggact tgtttacgtt ttcaggagaa aaaaaataac 720

tgtacccttt tcaatataga aataacattt gtagaaatcg tggattttcc ttaataaaca 780

atccaaaaca cgaccaccgt tgtctcctcg actcggtaac acccgatcgc cgacttgaaa 840

attagaagaa aaatgaaaag aataataaaa aaaaaaaagg aatgatgatt gaagctgtca 900

tatatgtcga ccctatcaca gtcaatccaa tagcctatat tcgccaactg atatatccaa 960

cggctcacaa attttcacaa acttttcaaa aaagtataat aaaagaggct gtctgacagc 1020

catgtcacgt tatacttttt ccgtatgatc gaaatgattc gtctttgyyg aatttaatta 1080

tttccaaaat tgaygactct aaagaaaaaa aaatagtttt tcagataaac ccgcctatat 1140

aaatagttca acactcggtt tatttcttct cccctcaaag aattgcctcg tcgtcttcag 1200

cttcatcggc cgttgcattt cccggcgata agagagagaa agaggagaaa gagtgagcca 1260

gatcttcatc gtcgtggttc ttgtttcttc ctcgatctct cgatcttctg cttttgcttt 1320

-continued

tccgattaag gtaattaaaa cctccgatct acttgttctt gtgttggatc tcgattacga 1380

tttctaagtt accttcaaaa gttgtttccg atttgatttt gattggaatt tagatcggtc 1440

aaactattgg aaatttttga tcctggcacc gattagctca acgattcatg tttgacttga 1500

tcttgcgttg tatttgaaat cgatccggat cctttcgctt cttctgtcaa taggaatctg 1560

aaatttgaaa tgttagttga agtttgactt cagattctgt tgatttattg actgtaacat 1620

tttgtcttcc gatgagtatg gattcgttga aatctgcttt cattatgatt ctattgatag 1680

atacatcata cattgaattg aatctactca tgaatgaaaa gcctggtttg attaagaaag 1740

tgttttcggt tttctcgatc aagattcaga tctttatgtt tttgattgca gatcgtagac 1800

<210> SEQ ID NO 30
<211> LENGTH: 1742
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1742)
<223> OTHER INFORMATION: chimeric promoter fusion CaMV and Act2
polynucleotides + Act2 intro

<400> SEQUENCE: 30

gtccgatgtg agacttttca acaaagggta atatccggaa acctcctcgg attccattgc 60

ccagctatct gtcactttat tgtgaagata gtggaaaagg aaggtggctc ctacaaatgc 120

catcattgcg ataaaggaaa ggccatcgtt gaagatgcct ctgccgacag tggtcccaaa 180

gatggacccc cacccacgag gagcatcgtg gaaaaagaag acgttccaac cacgtcttca 240

aagcaagtgg attgatgtga tggtccgatg tgagactttt caacaaaggg taatatccgg 300

aaacctcctc ggattccatt gcccagctat ctgtcacttt attgtgaaga tagtggaaaa 360

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-continued

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ga                                                                  1742
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We claim:

1. A DNA construct comprising: a first expression cassette, a second expression cassette, and a third expression cassette, wherein said first expression cassette in operable linkage comprises (i) a Figwort mosaic virus 35S promoter; (ii) a heterologous 5' untranslated leader; (iii) a chloroplast transit peptide DNA sequence; (iv) a DNA sequence encoding a glyphosate tolerant EPSPS; and (v) a transcriptional terminator; and said second expression cassette comprising in operable linkage (a) an Arabidopsis Elongation factor 1α promoter with homologous EF1α intron; (b) a chloroplast transit peptide DNA sequence; (d) a DNA sequence encoding a glyphosate tolerant EPSPS; and (e) a transcriptional terminator; and said third expression cassette comprising in operable linkage (I) an Arabidopsis Act2 promoter with homologous Act2 intron; (II) a chloroplast transit peptide DNA sequence; (III) a DNA sequence encoding a glyphosate tolerant EPSPS; and (IV) a transcriptional terminator.

2. A plant comprising the DNA construct of claim 1.

3. A plant of claim 2, wherein said plant is tolerant to glyphosate.

4. A plant of claim 3, wherein said plant is a dicot plant.

5. A method of controlling weeds, the method comprising: (1) providing a transformed crop plant with a DNA construct of claim 1; (2) selecting said transformed crop plant for tolerance to glyphosate herbicide; and (3) applying to the field in which the transformed crop plant is being grown a sufficient amount of glyphosate to inhibit weed growth without affecting the growth of said transformed crop plant.

* * * * *

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* see form PCT/ISA/210 (second sheet)	
Applicant's or agent's file reference see form PCT/ISA/220	**FOR FURTHER ACTION** See paragraph 2 below
International application No. PCT/US2006/020323	International filing date *(day/month/year)* 25.05.2006
Priority date *(day/month/year)* 27.05.2005	
International Patent Classification (IPC) or both national classification and IPC INV. A01H5/10 C12Q1/68	
Applicant MONSANTO TECHNOLOGY LLC	

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	see form PCT/ISA/210	Holtorf, Sönke Telephone No. +31 70 340-2627

Form (PCT/ISA/237) (Cover Sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2006/020323

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

      ☒ a sequence listing

      ☒ table(s) related to the sequence listing

   b. format of material:

      ☒ on paper

      ☒ in electronic form

   c. time of filing/furnishing:

      ☐ contained in the international application as filed.

      ☐ filed together with the international application in electronic form.

      ☒ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-25
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations

see separate sheet

1. The following documents are considered relevant for the current application:

D1: Terry, C., et al., Eur. Food Research and Technology (2001), 213, 6, 425-431
D2: WO0244407
D3: WO2004/072235
D4: Windels, P., et al., Eur. Food Research and Technology (2001), 213, 2, 107-112
D5: SUMMARY OF THE DOSSIER EFSA GMO NL 2006-36, Monsanto

Re Item V.

2. Novelty and **Clarity** (Art. 33(2) and Art. 6 PCT)

2.1 The current application is dealing with the generation of a transgenic herbicide-resistant soybean (*Glycine max*) plant that recombinantly expresses a herbicide-tolerant EPSPS gene from *Agrobacterium tumefaciens*.
The transgene is the EPSPS-gene which is responsible for mediating Glyphosate Herbicide Resistance; Glyphosate is toxic due to the inhibition of the shikimic acid pathway which is leading to aromatic amino acids.
A specific expression construct harbouring the *Agrobacterium tumefaciens* strain CP4-specific EPSPS gene operably linked to 5'-regulatory sequences consisting of enhancer sequences of the 35S promoter of the Figwort Mosaic Virus and the promoter of the *A. thaliana*-specific Tsf1-gene (Elongation factor EF-1alpha) was constructed. The 3' end is characterized by the Rubisco E9 sequence. A specific transformation/integration event MON89788 was identified and the specific integration sites (transgene/genome junctions) in the soybean genome were determined and are characterized by SEQIDs 1,2,3,4.
Specific primers (SEQIDs 5 and 6) were engineered to amplify a specific diagnostic amplicon in a PCR-assay to detect this certain transformation event MON89788 in progeny of the cross between this transgenic plant and non-transgenic plants.

Later published evidence (see T-Doc) discloses that the Event MON89788 of the current

application exhibits a 4-7% yield advantage compared to a previously obtained Glyphosate-resistant soybean Event 40-3-2 (compare Windels et al.,)

2.2 As currently drafted, claims 1-25 refer to the specific nucleic acid sequences as characterized by SEQIDs 1,2,3,4, the specific soybean plants and parts thereof that are characterized by or comprise said event MON89788 and to polynucleotide primers, detection kits and methods of detecting said transgenic event MON89788. Moreover, methods for producing a Glyphosate-tolerant soybean plant by crossing to a plant comprising the MON89788 event are claimed.
It appears that due to the fact that transgene-insertion in general is brought about by a random insertion process and due to the fact that every insertion event that is generated by this random process appears to be unique and not repeatable, all claims relating to the event MON89788 of the current application appear to be novel over the prior art.

2.3 Doubts exist about claims relating to "commodity products" obtained from soybean, like flour and oil. See current claims 4,5,11.

There seems to be no difference between a certain starch obtained from soybean when compared to any other starch with the same chemical characteristics which is obtained from another crop species. The same objection applies for e.g. oil.
The only difference between soybean starch obtained from wild type plants and starch obtained from the soybean plant characterized as "event MON89788" is the presence of residues of polynucleotides that could give rise to an amplicon of the right size when an amplification with specific primers is carried out.
It is questionable, what the difference between the actual starches should be. What about contaminations of starches from other plant species with residues originating from soybean MON89788-starch ?
The CP4/EPSPS transgene is responsible for mediating Glyphosate resistance. No other phenotypic changes in comparison to other soybean plants were detected, the current specification is silent about any advantage over existing Glyphosate-tolerant soybean plants (e.g. 40-3-2).

2.4 The subject matter of claims 4, 5 and 11 further relates to "commodity"-products being defined in terms of processes for their preparation ("product-by-process" claims).
Claim 11 is further drafted as a result to be achieved by relating to "a product comprising a nucleic acid that produces an amplicon when tested in a DNA amplification method". The same objection applies for claims 12 and 13 ("useful in a DNA amplification method") and claim 18 ("...primer set, which when used in a nucleic acid amplification reaction ....produces a diagnostic amplicon") and claim 24 ("treating the field with an amount of Glyphosate effective to control the growth...").
See also the definition of the soybean plant of claim 9 which is defined by its genome that "produces an amplicon diagnostic for event MON89788 when tested in a DNA amplification method". Claim 9 and claim 10 rather seem to be defining a diagnostic detection method rather than a soybean plant.

2.5 The "event MON89788" should be always defined by the respective SEQIDs. Page 3 of the current specification teaches that other abbreviations and codes are used for the same integration event, namely MON19788 and GM_A19788.

2.6 Claim 19 is very broad by relating to any "nucleic acid corresponding to event MON89788". Claim 21 refers to a soybean plant "comprising a glyphosate tolerant trait that is genetically linked to a nucleic acid molecule comprising SEQID1 or SEQID2". Apart from leaving it open to the reader which known or future "glyphosate tolerant trait" is meant, the wording "genetically linked" creates ambiguity.

3. Inventive Step (Art.33 (3) PCT)
3.1 Document D1 (Terry, C., et al, 2001) is the closest prior art and discloses the event-specific detection of RoundupReady (RR) soybean and the discrimination of RR soybean plants that have different transgene integration sites. Amplification of the integration border was performed based on the known 5' and 3' sites of integration of event 40-3-2 (RR soya); amplicons of different sizes are amplified.

The difference between D1 and the current application is the provision of another soybean plant transformed with an expression cassette comprising the CP4/EPSPS gene which is characterized by a specific integration event.
The problem of the current application is the provision of another specific transgenic soybean integration event that exhibits the desired best performance (yield).
The solution is the specifically selected integration event MON89788 characterized by a specific junction between the EPSPS-expression cassette's 5'- and 3'- ends and the surrounding genome.

3.2 Methods for detecting single specific insertion events in soybeans transformed with a construct comprising the CP4/EPSPS gene have already been disclosed in the prior art. See D1. It appears that the specific transgenic soybean line MON89788 is herbicide resistant. There is no experimental data provided, however, which would indicate the difference in yield-performance of event MON89788 over event 40-3-2. What is the achievement over the prior art apart from the fact that MON89788 is just another Glyphosate resistant soybean plant. The identified problem has not been solved and therefore inventive step cannot be assessed.
It is not clear whether an inventive step could be acknowledged even if experimental data were provided. The mere repetition of already existing protocols, i.e. the provision of yet another soybean plant transformed with already existing expression cassettes and the testing of the generated transgenic lines for the best agricultural performance seems obvious.

It is commonly known that the position of the integration site of the transgene in the host genome can have a profound effect on the expression pattern of the transgene ("position effect"). Moreover, it is common practice to look for a transgenic plant that only contains one single integration event (single copy transgene).

To circumvent any detrimental effect of the integration site on the expression ("position effect"), the person skilled in the art usually generates hundreds to thousands of transgenic plants followed by the routine screening of the resulting transgenic plants for the event with the highest expression and the best tissue-specific expression for

commercial purposes.

It is further known in the prior art how to characterize the precise locus of transgene integration and how to design specific primer pairs to amplify a specific nucleotide fragment that is unique for the certain integration event. See document D1.

The further identification of the specific insertion event and the specific junction regions of event MON89788 is general laboratory practice and so is the provision of respective SEQIDs for the junction region and potential primers useful to amplify a diagnostic amplicon.
Consequently, it appears that the subject matter is lacking an inventive step pursuant to Art.33 (3) PCT and it is not clear which part of the application could form the basis for a new allowable claim.

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XS CPRTENFRDE

BNSDOCID: <XS_____2006100103CF_I_>

272

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* MONS:113WO

Box No. I TITLE OF INVENTION

SOYBEAN EVENT MON89788 AND METHODS FOR DETECTION THEREOF

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MONSANTO TECHNOLOGY LLC
800 North Lindbergh Blvd.
St. Louis, MO 63167
US

Telephone No.

Facsimile No.

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MALVEN, Marianne
800 North Lindbergh Blvd.
St. Louis, MO 63167
US

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HANSON, Robert E.
FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, TX 78701
US

Telephone No. 512-474-5201

Facsimile No. 512-536-4598

Teleprinter No.

Agent's registration No. with the Office 42,628

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

RINEHART, Jennifer
800 North Lindbergh Blvd.
St. Louis, MO 63167
US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

TAYLOR, Nancy
800 North Lindbergh Blvd.
St. Louis, MO 63167
US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

DICKINSON, Ellen
800 North Lindbergh Blvd.
St. Louis, MO 63167
US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

   (i) *if more than two persons are to be indicated as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

   (ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

   (iii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

   (iv) *if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

   (v) *if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

2. *If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such a case, write the name or two-letter code of each designated State concerned and the indication "patent of addition," "certificate of addition," "inventor's certificate of addition" or "utility certificate of addition," the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other patent grant or the date of filing of the parent application (Rules 4.11(a)(iii) and 49bis.1(a) or (b)).*

3. *If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such a case, write "United States of America" or "US" and the indication "continuation" or "continuation-in-part" and the number and the filing date of the parent application (Rules 4.11(a)(iv) and 49bis.1(d)).*

U.S. Continutation-in-Part - U.S. Serial No. 60/685,584 filed 27 May 2005 (27.05.05)

Box No. V DESIGNATIONS

The filing of this request **constitutes under Rule 4.9(a), the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents. However,

☐ DE Germany **is not designated** for any kind of national protection
☐ JP Japan **is not designated** for any kind of national protection
☐ KR Republic of Korea **is not designated** for any kind of national protection
☐ RU Russian Federation **is not designated** for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application. See the Notes to Box No. V as to the consequences of such national law provisions in these States).

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application:* regional Office	international application: receiving Office
item (1) 27 May 2005 ~~27.05.05~~	60/685,584	US		
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*:

		Number of declarations
☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application **contains:**

(a) **on paper**, the following number of sheets:

request (including declaration sheets)	:	5
description (excluding sequence listing and/or tables related thereto)	:	26
claims	:	3
abstract	:	1
drawings	:	3
Sub-total number of sheets	:	38
sequence listing	:	10
tables related thereto	:	
(for both, actual number of sheets if filed on paper, whether or not also filed in electronic form; see (c) below)		
Total number of sheets	:	48

(b) ☐ **only in electronic form** (Section 801(a)(i))
- (i) ☐ sequence listing
- (ii) ☐ tables related thereto

(c) ☐ **also in electronic form** (Section 801(a)(ii))
- (i) ☐ sequence listing
- (ii) ☐ tables related thereto

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the
- ☐ sequence listing: .....
- ☐ tables related thereto: .....

(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)

This international application is **accompanied by** the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*: — Number of items

1. ☒ fee calculation sheet : 1
2. ☐ original separate power of attorney :
3. ☐ original general power of attorney :
4. ☐ copy of general power of attorney; reference number, if any: ..... :
5. ☐ statement explaining lack of signature :
6. ☐ priority document(s) identified in Box No. VI as item(s): ..... :
7. ☐ translation of international application into *(language)*: ..... :
8. ☐ separate indications concerning deposited microorganism or other biological material :
9. ☒ sequence listing in electronic form *(indicate type and number of carriers)* CD-R (1)
   - (i) ☒ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) :
   - (ii) ☐ *(only where check-box (b)(i) or (c)(i) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* :
   - (iii) ☒ together with relevant statement as to the identity of the copy or copies with the sequence listing mentioned in left column :
10. ☐ tables in electronic form related to sequence listing *(indicate type and number of carriers)*
    - (i) ☐ copy submitted for the purposes of international search under Section 802(b-*quater*) only (and not as part of the international application) :
    - (ii) ☐ *(only where check-box (b)(ii) or (c)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Section 802(b-*quater*) :
    - (iii) ☐ together with relevant statement as to the identity of the copy or copies with the tables mentioned in left column :
11. ☐ other *(specify)*: ..... :

Figure of the drawings which should accompany the abstract:

Language of filing of the international application:

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Robert E. Hanson, Agent

For receiving Office use only

1. Date of actual receipt of the purported international application:

2. Drawings:
   - ☐ received:
   - ☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

272

Apostille

(Convention de La Haye du 5 octobre 1961)

1. Country: United States of America

This public document

2. has been signed by CAITLIN E. MACAGY
3. acting in the capacity of NOTARY PUBLIC
4. bears the seal/stamp of CAITLIN E. MACAGY-NOTARY PUBLIC-STATE OF MISSOURI

Certified

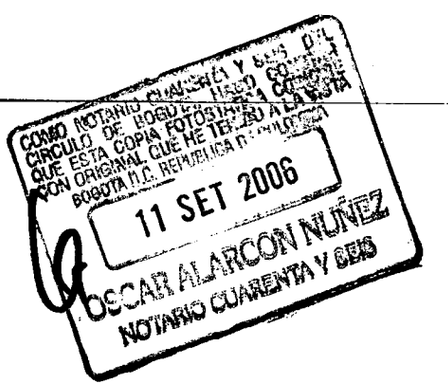
5. at Jefferson City, Missouri
6. the 28th. day of JANUARY, 2002.
7. by Matt Blunt, Secretary of State, State of Missouri
8. No. 70695
9. Seal/Stamp



10. Signature:

Matt Blunt

Comm. 48 (12-2000)



COLOMBIA

278

PODER

El abajo firmado

Nombra por el presente a

a los apoderados que él o ellos designen nuestro representante legal y verdadero en Bogotá, con facultades para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales, con el fin de dar cumplimiento al párrafo lo, del Articulo 543 del Decreto 410 de 1971, quedando especialmente facultados para representarnos ante las autoridades Colombianas, administrativas, judiciales o de policía, en todos los trámites relativos a la Propiedad Industrial, a cuyo efecto les facultamos para dar ante dichas autoridades todos los pasos necesarios al objeto indicado; elevar solicitudes, declaraciones y reclamos; formular descripciones, enmiendas; oposiciones y apelaciones; instaurar cancelaciones; abonar todos los impuestos y cuotas y efectuar todo otro pago determinado por la ley; recibir todos los documentos y valores, dando el descargo respectivo; llenar cualesquiera otros requisitos y tomar, en fin, todas las medidas que creyen conducentes al resguardo de nuestros intereses; y en caso de producirse oposición, pasar los antecedentes a los tribunales, quedando facultado, para intervenir como demandante o demandado ante los jueces que sean competentes, pudiendo transar, someter a arbitros, desistir, percibir y apelar, con todas las demás facultades que resulten necesarias, y por el presente declaramos desde ahora válido y bueno cuanto dicho representante y/o los apoderados que el nombre hicieren en nuestro beneficio.

Asimismo, facultamos a los abogados arriba indicados para solicitar en la Oficina de Propiedad Industrial la protocolización de este documento y de cualquier poder que otorguen a nombre nuestro.

POWER OF ATTORNEY

The undersigned MONSANTO TECHNOLOGY LLC, a Delaware Limited Liability company, located at 800 North Lindbergh Boulevard, St. Louis, Missouri, United States of America

hereby appoint Ramiro Castro-Duque y/o Juan Pablo Cadena Sarmiento y/o Beatríz Jaramillo S.

or the agents appointed by him or them, our true and legal representative in Bogota, with power to receive notifications and to appoint judicial or extrajudicial agents, so as to comply with paragraph 1, Article 543 of Decree 410 of 1971, and they are especially empowered to represent us before the administrative, judicial or police authorities of Colombia in all proceedings pertaining to industrial property, for which purpose we authorize them to take before the said authorities all the necessary steps to the said end; to file petitions, declarations, claims, cancellations, and administrative injunction proceedings; to draw up specifications; to make amendments, oppositions and appeals, to pay all installments and effect all other payments prescribed by law; to receive proper receipts, to comply with all other requirements and, in short, to take all those measures which may deem fit for the protection of our interests; and in case of opposition, where the proceedings be passed over to the courts, we authorize said agent(s) to act on our behalf, as complainant(s) as well as defendant(s), before the competent judges, with full powers to make arrangements, to submit to arbitrators, to desist, to collect, to appeal and, in short, with all such further powers as may be required; and we hereby declare from now on as valid and good all that said representative and/or the agents by him appointed may do for our benefit.

We also authorize said attorneys to apply for protocolization of this document and any power of attorney by him conferred in our name, before the Office of Industrial Property, as well as to substitute these presents.

Given and signed at (Dado y firmado en) St. Louis, Missouri, USA

this (a los) 9th day of(dias de) January de (of) 2006

MONSANTO TECHNOLOGY LLC

Por (By) Barbara Bunning-Stevens

Titulo (Title) Barbara Bunning-Stevens, Intellectual Property Counsel

COPIA DE ESTA COPIA FOTOSTATICA QUE PRESENTO ... CON ORIGINAL QUE PRESENTO ... BOGOTA D.C. REPUBLICA DE COLOMBIA

11 SET. 2006

OSCAR ALFONSO VARGAS VELEZ
NOTARIO CUARENTA Y SEIS

NOTARIAL CERTIFICATE (CORPORATION)

CERTIFICADO NOTARIAL (SOCIEDAD)

229

Estados Unidos de América)
Estado de) SS
Condado de)

United States of America)
State of MISSOURI) SS
County of ST. LOUIS)

Hoy de de 20 , compareció , a quien conozco y de

On this 9th day of January of 20 02 personally came

Barbara Bunning-Stevens

quien me consta que firmó el anterior documento, y quien debidamente juramentado declaró y dijo que él es

to me known, and known to me to be the person who signed the foregoing instrument, who being by me duly sworn did depose and say that she is the Intellectual Property Counsel

de

of Monsanto Technology LLC

la compañía nombrada en el documento, que antecede, una sociedad debidamente organizada y esixtente conforme a las leves del Estado de

Estados Unidos de America, domiciliada en la ciudad de
Condado de
Estado de , Estados Unidos de América y legalmente constituida para un período de duración que aún no ha determinada; que el declarante está autorizado por dicha Compañía para conferir este poder; que el sello estampado por él al pie de tal poder, en nombre y en representación de la expresada Compañía, es el sello oficial de ella; y que el objeto para el cual se confiere este instrumento está comprendido dentro del de la sociedad.

the company named within, a limited liability company organized and existing under the laws of the State of Delaware, United States of America, domiciled at the City of Creve Coeur

County of St. Louis
State of Missouri
United States of America, and legally constituted for a period of tenure not yet expired; that the deponent is authorized by said Limited Liability Company to confer this power of attorney; that the seal affixed hereunto by the deponent in the name and on behalf of said Limited Liability Company is the seal thereof; and that the purpose for which this instrument is granted is included within the corporate purposes of the Limited Liability Company.

El suscrito notario ha visto los estatutos de incorporación firmados en
Estado de
con fecha , y certifica que de conformidad con la legislación de dicho Estado la anterior declaración juramentada es prueba suficiente de que los hechos sobre que ella versa son ciertos o verdaderos, de lo cual da de.

The undersigned Notary Public has seen the Certificate of Formation of the said Limited Liability Company executed at Wilmington State of Delaware, under date of March 2, 2000 and hereby certifies that under the laws of said State the foregoing acknowledgment is sufficient proof that the facts therein contained are true, which she attests.

(Seal)

CAITLIN E. MACAGY
St. Louis County
My Commission Expires
January 21, 2005

Notary Public
(Notario Público)

ESTE NOTARIO CUARENTA Y SEIS DEL CIRCUITO DE BOGOTA HACE CONSTAR QUE ESTA COPIA FOTOSTATICA CONCUERDA CON ORIGINAL QUE HE TENIDO A LA VISTA
BOGOTA D. REPUBLICA DE COLOMBIA
11 SET 2006
OSCAR ALARCON NUÑEZ
NOTARIO CUARENTA Y SEIS

/2

REPÚBLICA DE COLOMBIA

GAVIRIA

ORGANIZACION ELECTORAL
REGISTRADURIA NACIONAL DEL ESTADO CIVIL

REGISTRO CIVIL DE DEFUNCION

Indicativo Serial 5562241

Datos de la oficina de registro

Clase de oficina: Registraduría [ ] Notaría [X] Consulado [ ] Corregimiento [ ] Insp. de Policía [ ] Código 979

País - Departamento - Municipio - Corregimiento o Inspección de Policía

COLOMBIA CUNDINAMARCA BOGOTA D.C. NOTARIA 32.

Datos del finado

Apellidos y nombres completos

CASTRO DUQUE RAMIRO

Documento de identificación (Clase y número)

C.C. No. 170322 DE BOGOTA

Sexo (en letras)

MASCULINO

Datos de la defunción

Lugar de la Defunción: País - Departamento - Municipio - Corregimiento o Inspección de Policía

COLOMBIA CUNDINAMARCA BOGOTA D.C.

Fecha de la defunción: Año 2004 Mes 10 Día 30

Hora: 04:10 PM

Número de certificado de defunción: A-a 2094375

Juzgado que profiere la sentencia

Presunción de muerte

Fecha de la sentencia: Año Mes Día

Documento presentado

Autorización judicial [ ] Certificado Médico [X]

Nombre y cargo del funcionario

ANDRES DIAZ CAMPOS
R.M. No. 79982662

Datos del denunciante

Apellidos y nombres completos

EDGAR DAVID REGINO H.

Documentos de identificación (Clase y número)

C.C. No. 15.030.154 DE LORICA/ CORDOBA

Firma

Primer testigo

Apellidos y nombres completos

Documentos de identificación (Clase y número)

Firma

Segundo testigo

Apellidos y nombres completos

Documentos de identificación (Clase y número)

Firma

Fecha de inscripción: Año 2004 Mes 1 Día 02

Nombre y firma del funcionario que autoriza

BLANCA LINDA CARDENAS RESTREPO
NOTARIA 32

PADRES: ALFONSO CASTRO MERCEDES DUQUE

ESPACIO PARA NOTAS

DECADA

NOTARIA 32
DEL CIRCULO DE SANTA FE DE BOGOTA, D.C.

Germán Eleázar Moreno L.
Notario

COMO SECRETARIO DELEGADO POR LA NOTARIA TREINTA Y DOS (32) DEL CIRCULO DE BOGOTA

CERTIFICO QUE:

La presente acta es fiel copia del original tomada del registro civil de defunción serial indentiva número: 5776291 que expedida en la ciudad de Bogotá D.C. hoy: 05 NOV. 2004

NELSON FLOREZ G.
SECRETARIO DELEGADO PARA COTEJAR
DECRETO 1334 DE 1986

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-136194- -00000-0000

Fecha: 2007-12-26 16:18:44 Dep: 2020 NUECREACIONI
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 280

Industria y Comercio
SUPERINTENDENCIA

REQUISITOS MINIMOS PARA ADMISION A TRAMITE PCT

PATENTE DE INVENCION [X] MODELO DE UTILIDAD [ ]

- Indicación de que se solicita la concesión de una patente [X]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [X]
- Descripción de la invención [X]
- Dibujos de ser estos pertinentes [ ]
- Comprobante de pago de las tasas establecidas [X]

COMPLETA [ ] INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Indicación de que se solicita el registro de Diseño Industrial [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

ESQUEMA DE TRAZADO [ ]

- Indicación de que se solicita el registro de un Esquema de Trazado [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica del esquema trazado [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

Claudia M Nova Cortes
Firma Responsable

Fecha_____

Carrera 13 No. 27-00 Piso 5, 7 y 10
Conmutador: 3 82 08 40
Fax: 350 52 20
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia